Post-traumatic stress disorder

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Epigenetic signatures of differential susceptibility to combat trauma

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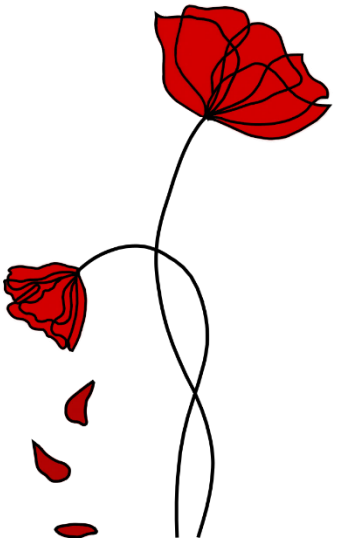
CHAPTER 1

General introduction



"*I started drinking a lot and being angry all the time but I didn't do anything about it. I didn't think it was an issue because everybody else in my unit was dealing with the same. One night I really freaked out, I had this feeling in my chest and it felt like it wouldn't go away. I looked at myself in the mirror and felt really scared. I felt a lot of guilt. So I chugged a beer, broke the bottle and started being really angry at myself. I slashed my chest multiple times. After that, I went back to my unit, but tried to hide it from my fellow soldiers. One day I asked if I could talk to one of the guys, and I told him "hey, don't think that I'm a wuss, but I tried to end my life". And he told me to get help. The first thing that went through my head was that I didn't want to be labeled. I didn't want to be* that *guy because that's just how our culture is. It took me a while to get over that, but I went and got help, and started talking to other guys who went through the same things I did. It feels good to realize it's never too late to go talk to somebody."*
- Afghanistan veteran [1]

Post-traumatic stress disorder

What this soldier is experiencing is not uncommon among war veterans, and is likely an indication of post-traumatic stress disorder (PTSD). PTSD is a debilitating psychiatric disorder that is categorized as a trauma- and stressor-related disorder by the Diagnostic and Statistical Manual of Mental Disorders (5^{th} edition; DSM-5; [2]). In the general (American) population, the lifetime prevalence of PTSD has been estimated around 3.6% for males and 7.9% for females, although this increases to 6.2% for male veterans and 13.2% for female veterans [3].

In order for a clinician to establish a PTSD diagnosis, several criteria need to be met based on the DSM-5 [2]. Direct or indirect exposure to a traumatic or stressful event is a necessary, but not sufficient, requirement. Although most individuals who experience a traumatic event recover relatively rapidly, others will develop persistent symptoms including intrusive memories or nightmares related to the traumatic event, avoidance behavior, negative thoughts and mood, and hyperarousal [2]. Additionally, these symptoms must be present for at least one month following trauma exposure and have a significant impact on one's daily functioning. They cannot be the result of medication or substance use, although this is often co-occurring as a coping strategy [4]. Similarly, the disorder is often comorbid with other psychiatric disorders such as major depressive disorder and anxiety disorders [4].

Over the past years, increasing efforts have been made to unravel the biological underpinnings of PTSD. To date, malfunctions of several (neural) networks and a large number of genetic variants have been associated with PTSD, although most of these findings lack replication [5]. Some of the most robust findings include (i) immune activation [6], (ii) dysregulated cortisol levels (mostly decreased [7], although a few studies also found increased levels as reviewed by Zoladz and Diamond [8]), and (iii) a dysregulated hypothalamic-pituitary-adrenal (HPA) axis characterized by an overactive negative feedback mechanism [9]. In order to better understand the biological basis of differential susceptibility to trauma, and develop effective interventions aimed at preventing mental illness, much attention is also given to studying factors that contribute to resilient phenotypes. Resilience refers to one's ability to successfully cope with stressors by withstanding and overcoming rising challenges [10]. It is best studied through prospective designs, which allow researchers to investigate one's capacity to dynamically adapt to a stressful or traumatic situation. These past years, resilience research moved researchers away from the traditional disease-centered approach and asked questions aimed at understanding why some individuals suffer when faced with adversity, while others do not. This approach encourages one to identify strategies to promote and protect mental health, as opposed to searching for (pharmacological) treatments to alleviate already existing symptoms [11]. One important field of study that can help explain how different phenotypic outcomes arise upon an environmental impact such as adversity, is epigenetics.

Epigenetics

As the term implies, "epigenetics" refers to processes occurring "on top of" our DNA. After its first introduction in 1942 [12], it now refers to several mechanisms that can dynamically intervene with gene expression while leaving the DNA sequence itself unmodified, and can be triggered by environmental factors [13]. Via epigenetic mechanisms, environmental factors can induce long-term changes in gene expression, which can lead to aberrant phenotypes. As such, over the past years, it has become clear that next to genetic predispositions, epigenetic mechanisms are key in contributing to various stress-related phenotypes and illnesses [14]. Two of these mechanisms are highlighted in this thesis, namely DNA methylation and microRNAs (miRNAs).

DNA methylation

Cytosine DNA methylation is one of the most studied and well-understood epigenetic modifications, which involves the addition of a methyl group to the fifth carbon of the

cytosine ring to produce 5-methylcytosine (5-mC) [15]. This process is mediated by DNA methyltransferases (DNMTs), some of which ascertain the maintenance of inherited methylation patterns (DNMT1), while others are involved in *de novo* methylation (DNMT3a and -3b) [16]. DNA methylation most often occurs at cytosine-phosphate-guanine (CpG) dinucleotides, while CpG islands, regions across the genome consisting of a large number of CpG dinucleotide repeats, usually remain unmethylated [17]. The effect of DNA methylation depends on where exactly in the genome this modification takes place. Generally, methylation of CpG islands located within gene promoters is associated with gene silencing by preventing transcription factors to bind to the DNA.

The most commonly used method to analyze DNA methylation involves treating the DNA with sodium bisulfite, which translates an epigenetic mark to genetic information. Specifically, it converts unmethylated cytosines into uracil residues which are subsequently replaced by thymine in a polymerase chain reaction (PCR), while methylated cytosines remain unmodified [18]. This bisulfite-converted DNA can then be used as input to arrays where probes can hybridize with the methylated or unmethylated (i.e. thymine substituted) loci. From these assays, a raw, site-specific methylation index, or β value can then be calculated [19]. The most commonly used arrays are the BeadChips from Illumina, of which earlier versions could assess the methylation status of about 27k CpG sites across the genome. Today, more advanced versions include the HumanMethylation450 BeadChip which assesses over 450k CpG sites, and the latest EPIC BeachChip covering more than 850k sites genome-wide.

MicroRNAs

miRNAs are single-stranded, non-coding RNA molecules with an average length of 22 nucleotides. Transcription of a miRNA gene, i.e. either an intergenic unit or a collection of introns and fewer exons from (non-) coding genes, occurs predominantly by RNA polymerase II and results in a primary miRNA (pri-miRNA) [20]. This transcript is then processed into a precursor miRNA (pre-miRNA) before gaining its final conformation as a mature miRNA duplex. This fragment comprises two strands, a 5p and a 3p strand, of which one is usually degraded. The other so-called guide strand associates with Argonaute (AGO) proteins to form the functional RNA-induced silencing complex (RISC) [21]. The seed region of a miRNA is comprised of 2-8 nucleotides at the 5′ end, and is crucial for recognizing and binding to a complementary 3′ untranslated region (UTR) of a messenger RNA (mRNA). In most cases, this binding either triggers translational repression or mRNA deadenylation and finally degradation [22]. Although more research is needed to unravel the precise effects of miRNA-mRNA interactions, it is now known

that one miRNA can target several hundreds of mRNAs and alternatively, one mRNA can be targeted by many different miRNAs.

Circulating miRNAs are present in several biological fluids including blood, cerebrospinal fluid and urine, in which they are either bound to proteins such as AGO, or encapsulated within vesicles such as exosomes [23, 24]. Exosomes are small vesicles of ~40-150nm that carry a wide variety of proteins and nucleic acids and are involved in intercellular communication [25]. Quantification of miRNAs, either protein-bound or present within vesicles, usually occurs through microarrays, quantitative PCR or next generation sequencing (NGS) [26]. Sample preparation for small RNA sequencing involves the isolation of RNA, e.g. from biological fluids or specific vesicles, followed by library preparations, which include adapter ligations, reverse transcription and PCR amplification. This technology allows one to discover new miRNAs on top of the 1917 currently identified precursor miRNAs and 2656 mature miRNAs in humans (miRBase release 22) [27].

Epigenetics of PTSD

Given the crucial role of trauma exposure in the etiology of PTSD, in the past decade, PTSD research has expanded upon the available genetic research findings by investigating epigenetic mechanisms such as DNA methylation. To date, most of these studies focused on candidate genes, while far less followed an epigenome-wide approach which allows for an unbiased exploration of epigenetic variations in relation to a specific phenotype. A large number of candidate gene-studies reported epigenetic modifications of genes involved in the HPA axis, such as the nuclear receptor subfamily 3 group C member 1 (*NR3C1)* and FK506 binding protein 5 (*FKBP5)* [28-33]. Alternatively, findings reported by epigenome-wide association studies (EWAS) mostly point towards immune dysregulations [34-36] and highlight genes involved in neuronal functioning and synaptic plasticity [37, 38]. Although still emerging, there currently is little overlap between research findings, and most studies are limited by small sample sizes and poor statistical power [39]. Therefore, international consortia such as the Psychiatric Genomics Consortium (PGC) for PTSD aim to combine data from large epidemiological studies in order to facilitate robust discoveries [40]. Their EWAS working group specifically aims to identify epigenetic patterns of PTSD by meta-analyzing methylation data from several military and/or civilian cohorts [39, 41].

Several critical considerations regarding epigenetic studies in PTSD need to be made. First, studies assessing longitudinally collected epigenetic data, i.e. data collected prior to and following trauma exposure, are valuable for taking into account pre-existing differences, and could allow one to identify epigenetic signatures predictive of future response to trauma exposure. To date, two studies have aimed to do this using DNA methylation data, one of which is part of this thesis [42, 43].

Second, identified epigenetic profiles should be unrelated to the traumatic event itself. Cancelling out the impact of the trauma exposure itself in observational human studies can best be done by comparing individuals with PTSD to individuals who were exposed to comparable levels of trauma, but did not develop PTSD (i.e. individuals who are said to be more resilient).

Third, studies that aim to link several biological dimensions, e.g. by attempting to link physiological and neurobiological observations with blood-based epigenetic profiles, may be highly relevant in order to understand whether such profiles could supplement questionnaire-based scores for disorders as complex as PTSD. Furthermore, since most epigenetic work so far has been done using peripheral (blood) samples only, one could question how the obtained findings relate to the tissue of interest, i.e. the brain [33, 44-46]. One study aimed to assess associations between blood methylation levels of *SKA2*, a gene that has been linked to suicide risk [47, 48], and cortical thickness and psychiatric outcomes in trauma-exposed veterans [45]. The authors found that blood *SKA2* methylation levels were negatively linked to cortical thickness in frontal brain regions, and positively linked to PTSD symptom severity [45]. Such findings point to the possibility of using blood indices as markers for psychiatric (endo)phenotypes and neurobiological features, and specifically stimulate future research to explore links between epigenetic and neuroimaging data.

Fourth, studies aiming to investigate several epigenetic layers together could lead to a better understanding of their combined effect on gene expression [49]. In one study, although using a limited sample size, the authors identified several differentially expressed blood-based miRNAs between PTSD cases and controls [49]. They then analyzed gene expression and identified differentially expressed gene networks that had immune-related functions, which correlated with global differences in miRNA expression. Many of these genes had altered DNA methylation levels, which suggests that both epigenetic mechanisms may play a role in the immune-related dysregulations often seen in PTSD [49].

Finally, these past years, miRNAs have repeatedly been suggested to be good biomarker candidates due to their presence in biofluids and relative stability when bound to proteins or when encapsulated within extracellular vesicles such as exosomes [50]. Interestingly, recently developed technologies allow one to specifically isolate and analyze subtypes of exosomes based on the presence of certain membrane proteins [51]. Given that exosomes are able to cross the blood-brain barrier, several studies have targeted CD171, also known as the neural cell adhesion molecule L1 or L1CAM, as a membrane marker for neuronal origin since this protein is mostly expressed in brain tissue. To date, other than the findings reported in this thesis, only one study has looked at the miRNA content of human blood-based neuron-derived exosomes (NDEs), i.e. miRNAs within exosomes which are present in blood and exhibit L1CAM on their membrane [52]. Although this study looked for NDE miRNA profiles associated with Alzheimer's disease, it paves the way for future studies to build upon their methodologies and apply these to psychiatric disorders such as PTSD.

At this stage, the potential diagnostic value of miRNAs in PTSD seems promising, but requires much more work in terms of optimizing study conditions and assessing practical feasibility. Similarly, although it is unclear to what extent blood DNA methylation patterns accurately reflect methylation profiles within the brain, peripheral CpG sites could, if robust, prove to be informative as potential diagnostic biomarkers for PTSD.

Combat-related PTSD: the need for diagnostic biomarkers

Jobs during which exposure to potentially shocking events is almost inevitable, e.g. jobs within the military or police force, put individuals at increased risk of developing trauma-related disorders such as PTSD. However, as reflected by the opening section of this introduction, combat veterans or returning military members who suffer from mental health-related issues may not be willing to fully disclose their symptoms out of apprehension about "what others will say". Other commonly perceived barriers to seeking mental health care include career-related concerns and lack of support by other military members and leaders [53]. Additionally, the media often portrays veterans with PTSD as being angry, fragile and rather unstable individuals who experience difficulties in getting back to their daily lives. One can only imagine that veterans do not want to fall under that stereotyped category, which could further cause them to hide or minimize experienced symptoms. Given the high prevalence of PTSD among combat veterans, this is alarming, and needs to be addressed properly in order to encourage suffering individuals to seek appropriate treatment.

Over the past years, some efforts have been made to break through the stigma associated with combat-related PTSD and change the narrative surrounding mental health care [54]. Of note, although these efforts could also be useful for the civilian population, this stigma might be amplified in the military due to emphasized values such as physical and mental strength and everlasting endurance [55]. One of those efforts includes the (highly debated) proposition of redefining PTSD as an injury that can follow trauma exposure and can be reversed when treated [56]. That way, the focus would be taken away from seeing PTSD as a *mental* condition with predetermined risk factors. Alternatively, identifying (a set of) objective biological markers that could accurately reflect PTSD status could further pierce through any stigma-related issues, and legitimize the disorder by highlighting and emphasizing the undeniable biological dimension [55]. Moreover, as previously described by Yehuda and colleagues [57], disclosing one's symptoms requires a certain level of introspection which may not be straightforward for all. For these individuals, using such a marker as a complement to the symptom-based post-deployment diagnostic assessments, clinicians could indirectly confirm what cannot be put into words. It could also further minimize misdiagnosis which is common with PTSD given its high complexity and the high level of co-morbidities [58]. Together, this suggests that identifying diagnostic biomarkers for combat-related PTSD could hold promise for implementation within the clinic.

Aims and research questions of the thesis

This thesis aims to build upon the available literature by (i) examining how studying resilience factors could help us better understand differential susceptibility to traumatic experiences, (ii) identifying miRNAs and DNA methylation patterns associated with PTSD, and (iii) start exploring whether such epigenetic signatures could serve as diagnostic biomarkers for PTSD.

In order to address these aims, the following research questions were established:

1. What is known regarding resilience and traumatic stress and how can this be valuable for PTSD research?
2. What is known regarding the implications of microRNAs in PTSD?
3. What microRNAs and DNA methylation signatures are associated with combat-related PTSD?
4. Could the identified epigenetic signatures serve as diagnostic biomarkers of combat-related PTSD?

Outline of the thesis

The review presented in **Chapter 2** aims to shed light on the positive outcome of stress exposure, which is commonly referred to as resilience. As mentioned earlier, understanding the biological basis of differential susceptibility to trauma exposure could potentially allow one to develop more accurate preventive strategies for PTSD and protect mental health.

Chapter 3 gives an overview of what is known regarding circulating miRNAs in PTSD. This review points to a current lack of replication of study findings, and suggests strategies to move this field forward.

The pilot study presented in **Chapter 4** aims to discover circulating miRNAs associated with PTSD and explores the potential of using these as diagnostic biomarkers using blood samples belonging to a Dutch military cohort. Given the relative lack of replication with findings from similar studies and potential lack of specificity of these miRNAs, we went on to explore a similar avenue in following chapters, i.e. miRNAs within NDEs.

The optimized protocol needed to start examining miRNAs encapsulated within NDEs is presented in **Chapter 5**. This shorter methodological chapter aims to convey the knowledge gained throughout a year of protocol optimization and can serve as a working protocol for any interested researcher in the field.

Using the protocol described in Chapter 5, **Chapter 6** presents a pilot study aiming to indirectly gain insights into pathological mechanisms occurring within the brains of individuals with PTSD. Specifically, it assesses whether miRNAs within NDEs can be isolated from limited amounts of plasma, and potentially serve as markers of PTSD. Additionally, we examined whether the same protocol can be used on urine samples belonging to the same individuals, and on older serum samples (± 10 years old) from an independent PTSD cohort.

A different approach to identifying epigenetic dysregulations in PTSD was taken in **Chapter 7**, which presents the first meta-analysis of its kind. Specifically, the study performed in this chapter combines longitudinally collected DNA methylation data from three highly similar military cohorts in order to identify PTSD-associated CpG sites.

In the final chapter, **Chapter 8**, the key findings of this thesis are discussed along with overall strengths, limitations and suggestions for future research.

References

1. News, M.M. *Soldier talks about his struggle with depression and PTSD*. Youtube 2013 [cited 23 March 2020]; Available from: https://www.youtube.com/watch?v=4DF5caucKjI.
2. American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
3. Lehavot, K., et al., *Post-traumatic Stress Disorder by Gender and Veteran Status.* Am J Prev Med, 2018. **54**(1): p. e1-e9.
4. Gallagher, M.W. and T.A. Brown, *Bayesian Analysis of Current and Lifetime Comorbidity Rates of Mood and Anxiety Disorders In Individuals with Posttraumatic Stress Disorder.* J Psychopathol Behav Assess, 2015. **37**(1): p. 60-66.
5. Ashley-Koch, A.E., et al., *Genome-wide association study of posttraumatic stress disorder in a cohort of Iraq-Afghanistan era veterans.* J Affect Disord, 2015. **184**: p. 225-34.
6. Wang, Z., B. Caughron, and M.R.I. Young, *Posttraumatic Stress Disorder: An Immunological Disorder?* Front Psychiatry, 2017. **8**: p. 222.
7. Pan, X., et al., *Salivary cortisol in post-traumatic stress disorder: a systematic review and meta-analysis.* BMC Psychiatry, 2018. **18**(1): p. 324.
8. Zoladz, P.R. and D.M. Diamond, *Current status on behavioral and biological markers of PTSD: A search for clarity in a conflicting literature.* Neuroscience & Biobehavioral Reviews, 2013. **37**(5): p. 860-895.
9. Yehuda, R., et al., *Post-traumatic stress disorder.* Nat Rev Dis Primers, 2015. **1**: p. 15057.
10. Kalisch, R., et al., *The resilience framework as a strategy to combat stress-related disorders.* Nat Hum Behav, 2017. **1**(11): p. 784-790.
11. Sapienza, J.K. and A.S. Masten, *Understanding and promoting resilience in children and youth.* Curr Opin Psychiatry, 2011. **24**(4): p. 267-73.
12. Waddington, C., *Canalization of development and their inheritance of acquired characters.* Nature, 1942. **150**: p. 563-565.
13. Felsenfeld, G., *A brief history of epigenetics.* Cold Spring Harb Perspect Biol, 2014. **6**(1).
14. Moosavi, A. and A. Motevalizadeh Ardekani, *Role of Epigenetics in Biology and Human Diseases.* Iran Biomed J, 2016. **20**(5): p. 246-58.
15. Moore, L.D., T. Le, and G. Fan, *DNA methylation and its basic function.* Neuropsychopharmacology, 2013. **38**(1): p. 23-38.
16. Bird, A., *DNA methylation patterns and epigenetic memory.* Genes Dev, 2002. **16**(1): p. 6-21.
17. Portela, A. and M. Esteller, *Epigenetic modifications and human disease.* Nat Biotechnol, 2010. **28**(10): p. 1057-68.
18. Li, Y. and T.O. Tollefsbol, *DNA methylation detection: bisulfite genomic sequencing analysis.* Methods Mol Biol, 2011. **791**: p. 11-21.
19. Pidsley, R., et al., *A data-driven approach to preprocessing Illumina 450K methylation array data.* BMC Genomics, 2013. **14**: p. 293.
20. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
21. Yoda, M., et al., *ATP-dependent human RISC assembly pathways.* Nat Struct Mol Biol, 2010. **17**(1): p. 17-23.

22. Huntzinger, E. and E. Izaurralde, *Gene silencing by microRNAs: contributions of translational repression and mRNA decay*. Nat Rev Genet, 2011. **12**(2): p. 99-110.
23. Arroyo, J.D., et al., *Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma*. Proc Natl Acad Sci U S A, 2011. **108**(12): p. 5003-8.
24. Turchinovich, A., et al., *Characterization of extracellular circulating microRNA*. Nucleic Acids Res, 2011. **39**(16): p. 7223-33.
25. Zhang, Y., et al., *Exosomes: biogenesis, biologic function and clinical potential*. Cell Biosci, 2019. **9**: p. 19.
26. Kolbert, C.P., et al., *Multi-platform analysis of microRNA expression measurements in RNA from fresh frozen and FFPE tissues*. PLoS One, 2013. **8**(1): p. e52517.
27. Kozomara, A., M. Birgaoanu, and S. Griffiths-Jones, *miRBase: from microRNA sequences to function*. Nucleic Acids Res, 2019. **47**(D1): p. D155-D162.
28. Klengel, T., et al., *Allele-specific FKBP5 DNA demethylation mediates gene–childhood trauma interactions*. Nature Neuroscience, 2013. **16**(1): p. 33-41.
29. Yehuda, R., et al., *Epigenetic Biomarkers as Predictors and Correlates of Symptom Improvement Following Psychotherapy in Combat Veterans with PTSD*. Frontiers in Psychiatry, 2013. **4**: p. 118.
30. Yehuda, R., et al., *Influences of Maternal and Paternal PTSD on Epigenetic Regulation of the Glucocorticoid Receptor Gene in Holocaust Survivor Offspring*. American Journal of Psychiatry, 2014. **171**(8): p. 872-880.
31. Labonté, B., et al., *Epigenetic modulation of glucocorticoid receptors in posttraumatic stress disorder*. Translational Psychiatry, 2014. **4**(3): p. e368-e368.
32. Yehuda, R., et al., *Lower Methylation of Glucocorticoid Receptor Gene Promoter 1F in Peripheral Blood of Veterans with Posttraumatic Stress Disorder*. Biological Psychiatry, 2015. **77**(4): p. 356-364.
33. Vukojevic, V., et al., *Epigenetic modification of the glucocorticoid receptor gene is linked to traumatic memory and post-traumatic stress disorder risk in genocide survivors*. J Neurosci, 2014. **34**(31): p. 10274-84.
34. Kuan, P.F., et al., *An epigenome-wide DNA methylation study of PTSD and depression in World Trade Center responders*. Translational Psychiatry, 2017. **7**(6): p. e1158-e1158.
35. Smith, A.K., et al., *Differential immune system DNA methylation and cytokine regulation in post-traumatic stress disorder*. Am J Med Genet B Neuropsychiatr Genet, 2011. **156B**(6): p. 700-8.
36. Uddin, M., et al., *Epigenetic and immune function profiles associated with posttraumatic stress disorder*. Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9470-5.
37. Uddin, M., et al., *Epigenetic meta-analysis across three civilian cohorts identifies NRG1 and HGS as blood-based biomarkers for post-traumatic stress disorder*. Epigenomics, 2018. **10**(12): p. 1585-1601.
38. Uddin, M., et al., *Epigenetic Signatures May Explain the Relationship between Socioeconomic Position and Risk of Mental Illness: Preliminary Findings from an Urban Community-Based Sample*. Biodemography and Social Biology, 2013. **59**(1): p. 68-84.

39. Ratanatharathorn, A., et al., *Epigenome-wide association of PTSD from heterogeneous cohorts with a common multi-site analysis pipeline.* Am J Med Genet B Neuropsychiatr Genet, 2017. **174**(6): p. 619-630.
40. Nievergelt, C.M., et al., *Genomic Approaches to Posttraumatic Stress Disorder: The Psychiatric Genomic Consortium Initiative.* Biol Psychiatry, 2018. **83**(10): p. 831-839.
41. Smith, A.K., et al., *Epigenome-wide meta-analysis of PTSD across 10 military and civilian cohorts identifies novel methylation loci.* bioRxiv, 2019: p. 585109.
42. Rutten, B.P.F., et al., *Longitudinal analyses of the DNA methylome in deployed military servicemen identify susceptibility loci for post-traumatic stress disorder.* Mol Psychiatry, 2018. **23**(5): p. 1145-1156.
43. Snijders, C., et al., *Longitudinal epigenome-wide association studies of three male military cohorts reveal multiple CpG sites associated with post-traumatic stress disorder.* Clin Epigenetics, 2020. **12**(1): p. 11.
44. Schechter, D.S., et al., *Methylation of NR3C1 is related to maternal PTSD, parenting stress and maternal medial prefrontal cortical activity in response to child separation among mothers with histories of violence exposure.* Front Psychol, 2015. **6**: p. 690.
45. Sadeh, N., et al., *SKA2 methylation is associated with decreased prefrontal cortical thickness and greater PTSD severity among trauma-exposed veterans.* Mol Psychiatry, 2016. **21**(3): p. 357-63.
46. Wolf, E.J., et al., *Accelerated DNA methylation age: Associations with PTSD and neural integrity.* Psychoneuroendocrinology, 2016. **63**: p. 155-62.
47. Guintivano, J., et al., *Identification and replication of a combined epigenetic and genetic biomarker predicting suicide and suicidal behaviors.* Am J Psychiatry, 2014. **171**(12): p. 1287-96.
48. Niculescu, A.B., et al., *Psychiatric blood biomarkers: avoiding jumping to premature negative or positive conclusions.* Mol Psychiatry, 2015. **20**(3): p. 286-8.
49. Bam, M., et al., *Dysregulated immune system networks in war veterans with PTSD is an outcome of altered miRNA expression and DNA methylation.* Sci Rep, 2016. **6**: p. 31209.
50. Wang, J., J. Chen, and S. Sen, *MicroRNA as Biomarkers and Diagnostics.* J Cell Physiol, 2016. **231**(1): p. 25-30.
51. Mustapic, M., et al., *Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes.* Front Neurosci, 2017. **11**: p. 278.
52. Cha, D.J., et al., *miR-212 and miR-132 Are Downregulated in Neurally Derived Plasma Exosomes of Alzheimer's Patients.* Front Neurosci, 2019. **13**: p. 1208.
53. Hoge, C.W., et al., *Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care.* N Engl J Med, 2004. **351**(1): p. 13-22.
54. Johnson, H.P. and M. Agius, *A Post-Traumatic Stress Disorder review: the prevalence of underreporting and the role of stigma in the Military.* Psychiatr Danub, 2018. **30**(Suppl 7): p. 508-510.
55. Lehrner, A. and R. Yehuda, *Biomarkers of PTSD: military applications and considerations.* Eur J Psychotraumatol, 2014. **5**.
56. Sagalyn, D., *Key Psychiatric Doctor Rejects Name Change for PTSD*, in *PBS News Hour*. 2010.
57. Yehuda, R., et al., *The use of biomarkers in the military: from theory to practice.* Psychoneuroendocrinology, 2013. **38**(9): p. 1912-22.

58. Aspesi, D. and G. Pinna, *Could a blood test for PTSD and depression be on the horizon?* Expert Rev Proteomics, 2018. **15**(12): p. 983-1006.

CHAPTER 2

Resilience against traumatic stress: current developments and future directions

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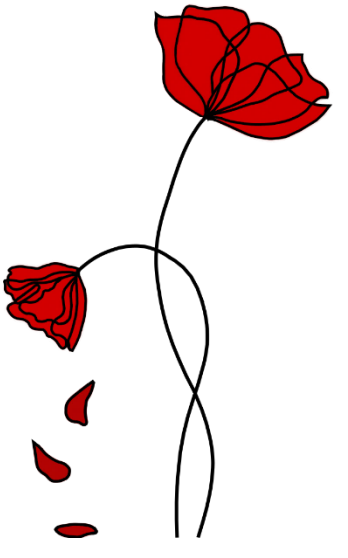

Abstract

Stress-related mental disorders are highly prevalent and have a significant impact on the suffering individual, their surroundings and society as a whole. Given the lack of effective treatment options, there is currently a pressing need for innovative approaches to address these issues. One approach that has received increasing attention over the last decade, is to shift our scientific and clinical focus from risk factors for psychopathology to factors promoting resilience and mental well-being. In order to summarize and evaluate the current state of scientific affairs on the biological basis of resilience, we provide an overview of the literature on animal and human studies of resilience. Because resilience can only truly be operationalized through longitudinal data collection and analysis, we focus on longitudinal studies only. This review shows that resilience is currently being defined and measured in several ways, both within animal and human studies. We further provide an overview of existing and new strategies that could help promote resilience. Finally, we summarize the challenges the field is facing and provide recommendations for future research.

Keywords: resilience, stress, prospective longitudinal studies, resilience-promoting interventions, review

Introduction

This past decade, research on resilience has received increasing attention. The heightened interest in understanding and promoting resilience is not surprising given that in Europe alone, anxiety disorders and major depressive disorder were among the most frequent mental health disorders in 2011 with a 12-month prevalence of 14% (corresponding to 61.5 million individuals) and 6.9% (30.3 million individuals), respectively [1]. Moreover, a recent meta-analysis showed that relapse rates in patients suffering from depression remain high and that long-term effects of conventional treatment options are not always encouraging [2]. These findings call for additional strategies and alternative interventions to prevent disease development and boost resilience.

The concept of resilience reflects the active and dynamic process through which a person adaptively overcomes a stressful or difficult situation. Resilience is not a passive reaction to an adverse situation, nor is it merely the reverse side of post-traumatic stress disorder (PTSD) or the absence of stress-related symptomatology [3, 4]. Research on resilience is facing several challenges of which the most fundamental one consists of the enormous heterogeneity in defining resilience. Therefore, the following proposals have recently been formulated in order to guide future resilience research: (i) resilience refers to the fast recovery of mental health following stress exposure and reflects a dynamic adaptation process, (ii) resilience should not be understood as a personality trait, the result of a specific genotype or other hardwired characteristics, (iii) resilience can only be studied following a stressful period or event, and (iv) there is a strong need for prospective longitudinal studies encompassing a period of stress exposure in order to better capture its dynamic nature [3].

The aim of this chapter is to summarize the findings from key longitudinal animal and human studies on resilience as well as to propose strategies aiming to promote resilience. Finally, we discuss the challenging nature of resilience research and suggest future directions to help this field evolve in the right direction.

Resilience Studies

Most published resilience studies to date used cross-sectional designs. However, susceptibility and resilience to past or ongoing stress are difficult to capture when assessing mental health at one time point only. Moreover, these designs do not allow for the assessment of baseline differences between individuals, which further impairs a

comprehensive interpretation of the obtained findings. Using a longitudinal design enables one to assess dynamic behavioral and biological fluctuations over time, while allowing for investigations of baseline predictors of differential susceptibility to future stress. This is highly relevant, especially in light of at-risk jobs in which trauma exposure is more prevalent.

The first part of this review provides an overview of prospective longitudinal animal and human studies, i.e. studies in which the mental health of the included subjects and/or behavioral or physiological states of animals were quantitatively assessed before stress exposure and at least once after such a period. Furthermore, we only included studies in which the severity and duration of the stress exposure was precisely quantified. In addition, and as postulated by Kalisch *et al.* (2017), in susceptible individuals, the severity of the stress exposure had to positively correlate with the development of mental health problems. Finally, studies were only considered when the study groups consisted of at least 30 subjects [3].

Animal Studies

In animal research, resilient phenotypes are often identified based on specific behavioral outcomes following a well-defined period of experimentally induced stress. Specifically, animals showing a fast recovery from a stressful manipulation are often said to be resilient. Although caution and critical evaluation of the observed phenotypes are needed when interpreting and translating the obtained findings, these models can provide us with some of the molecular underpinnings of differential susceptibility to stress. Applying our eligibility criteria led to the inclusion of five studies here. Studies sharing similar features are discussed together in the next paragraphs and are summarized in Table 1.

Corticosterone

The first study examined acoustic startle responses (which has been related to PTSD) in rats before and after a period of exposure to a single episode of inescapable footshocks and one-minute reminders for the next six weeks [5]. Rats exhibiting a high baseline startle response showed a significantly higher startle response following the period of stress exposure. Interestingly, rats showing an increased startle response at baseline exhibited elevated plasma corticosterone levels at follow-up as compared to the rats with low baseline startle responses (it should be noted that corticosterone levels were not measured prior to the experimental manipulation). Links between increased corticosterone levels and stress-induced behavioral phenotypes have also been observed in other studies [6, 7]. One of these studies investigated whether longitudinal changes in

blood corticosterone levels were associated with measures of differential susceptibility to stress in mice [6]. The authors found that mice with an increase in plasma corticosterone levels upon 2 weeks of repeated restraint stress also showed significant weight loss over the course of the experiment as well as anxiety-related behaviors at follow-up as measured through the Elevated Plus Maze (EPM) and Open Field Test (OFT). These mice were thus characterized as being susceptible while the more resilient mice were characterized by having (i) a decrease in corticosterone levels from baseline to follow-up, (ii) a stable body weight, and (iii) no anxiety-related behaviors at follow-up. Furthermore, the authors found that corticosterone levels at baseline predicted the extent of change in corticosterone levels during stress exposure and correlated with behavioral measures at follow-up. Together, these results suggest that baseline corticosterone levels might predict differential susceptibility to future stress exposure.

MicroRNAs

Only one prospective study examined the potential of microRNAs (miRNAs) to distinguish resilient animals from vulnerable animals [8]. miRNAs are small, non-coding RNAs which are involved in the post-transcriptional regulation of gene expression [9]. miRNAs have been widely studied in cancer and cardiovascular disease as potential disease biomarkers, but less is known regarding their involvement in mental health disorders. Chen *et al.* (2015) examined whether miRNAs could serve as biomarkers of resilience or vulnerability to stress by using a chronic social defeat paradigm in rats that lasted for 7 days. Rats showing little or no avoidance behavior when encountering an unfamiliar rat in its own cage were defined as resilient. Rats showing behavioral signs of susceptibility at follow-up showed lower baseline blood circulating levels of miR-24-2-5p, miR-27a-3p, miR-30e-5p and miR-362-3p compared to unexposed controls. However, rats not showing behavioral signs of susceptibility at follow-up, i.e. the resilient rats, had lower levels of blood circulating levels of miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p at follow-up as compared to controls. These results pinpoint a number of candidate miRNAs which, at least in part, can reflect vulnerability to future stress or reflect ongoing resilience to chronic social stress in rats.

Neuroimaging

Using magnetic resonance imaging (MRI) in mice, Tse *et al.* (2014) were the first to assess changes in hippocampal volume prior to and following stress exposure [10]. The authors identified susceptible and resilient groups based on their behavioral profiles in the social defeat paradigm. Approximately half of the mice was classified as susceptible to stress, while the other half was more resilient. In contrast to the susceptible mice which showed

no change in hippocampal volume over time, mice in the resilient and non-stressed control groups showed an increase in the left hippocampal volume from baseline to post-stress exposure. Intriguingly, a positive correlation was observed between hippocampal volume at baseline and social avoidance behavior at follow-up. This suggests that differences in hippocampal volumes could be associated with vulnerability to future stress in mice, which is further supported by similar findings in humans [11].

More recently, another study assessed structural changes in the brain more broadly, along with alterations in the brain's functional connectome upon stress exposure [7]. The mice in this study underwent a chronic unpredictable stress protocol for 3 weeks. Blood corticosterone levels, MRI scans and anxiety-related behaviors (measured through the EPM) were measured prior to and following the period of stress exposure. The authors categorized the stress-exposed mice into susceptible and resilient groups based on aberrant behavior and plasma corticosterone levels, i.e. mice expressing lower levels of post-exposure corticosterone in combination with less anxiety-like behavior were categorized as resilient. Among other structural and functional alterations induced by stress, the authors found that baseline differences in functional connectivity measures of a specific brainstem-limbic network were able to distinguish the resilient and susceptible groups, with susceptible rats showing lower functional connectivity compared to the resilient ones. It is worth mentioning that in humans, distinct patterns of brain activity have also been linked to PTSD [12-14]. Together, these results suggest that imaging data can contribute to a better understanding of the psychopathology of PTSD and potentially serve as a predictive biomarker of future vulnerability to stress.

Table 1. Longitudinal animal studies assessing biological outcomes associated with differential susceptibility to stress.

Species and sex	Stressor	Outcome of interest	Main finding
Male Sprague–Dawley rats	7 days of chronic social defeat stress	Circulating miRNAs (tail blood)	↓ pre-stress miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p, associates with future vulnerability to chronic social stress; ↓ post-stress miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p associates with ongoing resilience [8]
Male CD45.1+/CD 45.2+ C57BL/6 mice	10 days of repeated social defeat stress	Blood leukocytes and IL-6 levels	Higher pre-stress leukocyte levels in mice that later became stress vulnerable. Higher IL-6 levels following acute stress, only in those mice that later became stress vulnerable [15]
Male C57BL/6N mice	Chronic restraint stress	Plasma corticosterone levels	Longitudinal changes in corticosterone reflect differential stress susceptibility & pre-stress corticosterone predicts post-stress susceptibility or resilience [6]
Male Wistar rats	3 weeks of chronic unpredictable stress	Neuroimaging – functional connectivity and structural changes	Pre-stress differences in functional connectivity in brainstem-limbic area between susceptible and resilient rats [7]
Male Wistar rats	Inescapable footshock with weekly 1-min reminders for 6 weeks	Acoustic startle response	↑ pre-stress acoustic startle response = ↑ post-stress plasma corticosterone levels & ↑ post-stress acoustic startle response [5]
Male C57BL/6 mice	10 days of chronic social defeat stress	Hippocampal volume	↑ post-stress left hippocampal volume in resilient and control mice [10]

Studies are listed in alphabetical order based on the surname of the first author. IL-6: interleukin-6.

Human Studies

Various collaborative prospective approaches are currently being conducted, including the Prospective Research in Stress-Related Military Operations (PRISMO) [16-20], the Marine Resiliency Study (MRS) I and II [21-23], and the Prevalence, Incidence and Determinants of PTSD and Other Mental Disorders (PID-PTSD^{+3}) [24, 25]. Following our eligibility criteria, 13 studies were identified and incorporated in the present review. However, most of these are still preliminary since replication of findings in independent cohorts is often lacking, most harbor low effect sizes and relatively small sample sizes [3]. It is also important to note that (i) studies on 'resilience' use a wide variety of measurement tools of which validity is under discussion and which cannot be compared easily between studies, and (ii) the focus of the majority of studies was on PTSD-related outcomes and not on positive outcomes. The studies focusing on biological risk and resilience factors moderating stress-related mental health outcomes are reported in Table 2.

Genetic factors

Two studies examined genetic variations in stress susceptibility using longitudinal designs. Both studies focused on candidate genes, i.e. the serotonin transporter (*5-HTT*) gene and catechol-O-methyltransferase (*COMT*) gene, which affect serotonergic and dopaminergic signaling, respectively.

In the first study, associations between the serotonin transporter gene-linked polymorphic region (5-HTTLPR) and threat-related attention in post-deployment PTSD was evaluated in 1,085 male soldiers [26]. PTSD symptoms and threat-related attention bias (measured with a computerized dot-probe task) were assessed three times, with the last assessment taking place around one year after baseline. Combat exposure between baseline and follow-up assessments was inferred by using geo-operational exposure data and self-report measures (i.e. the Combat Experiences Scale with two additional items [27]). The authors observed that pre-deployment threat bias interacted with combat exposure during deployment and 5-HTTLPR in predicting post-deployment PTSD symptoms. More specifically, fewer post-deployment PTSD symptoms after high combat exposure were found in those individuals who displayed pre-deployment threat vigilance and had the SS/SL genotypes of 5-HTTLPR (reflecting low transcription 5-HTT). This study is particularly interesting in highlighting the complex interaction between stress-exposure, attention bias and genetic predisposition, suggesting that serotonergic transmission may be involved in the co-occurrence of avoidance and hypervigilance symptoms in PTSD [28].

In another study, 253 Iraq war veterans were assessed prior to and following a deployment period of 16 months [29]. Deployment trauma was measured with the Post Deployment Stressors subscale of the Deployment Risk and Resilience Inventory (DRRI) [30] as well as by using one additional item on sexual assault experienced during deployment. DNA was extracted from blood or buccal swabs and was genotyped into COMT Met/Met (N=63), Val/Met (N=131), or Val/Val (N=42). Regression analyses showed that the effect of deployment trauma on PTSD was dependent on COMT polymorphism with carriers of the homozygous genotypes (Met/Met and Val/Val) showing more PTSD symptoms than those carrying the heterozygous (Val/Met) genotype. This is in line with previous human and animal studies, highlights the role of the Met/Met genotype and show preliminary support for the Val/Val genotype as a risk factor for the development of PTSD [31-33].

Epigenetic mechanisms

Several cross-sectional studies support the role of epigenetic mechanisms, especially DNA methylation, in the impact of traumatic stress on mental health [34-37]. Recently, prospective epigenetic studies started to investigate links between changes in PTSD symptoms and changes in epigenetic profiles across a period of trauma exposure. These studies were conducted using subsamples of the PRISMO project and focus on the glucocorticoid receptor exon 1_F (GR-1_F) region and the predictive role of epigenetic markers in PTSD. In the first study, methylation signatures of the GR-1_F region (52 loci) were quantified in peripheral blood cells of 92 Dutch military members whose blood was collected before and after a four-month deployment period to Afghanistan. Increased methylation at post-deployment was associated with mental health. Baseline methylation at this site could not predict future PTSD or mental health [17].

In a recent prospective epigenetic study using two military cohorts [16], the impact of traumatic stress during combat on post-deployment PTSD symptoms and longitudinal epigenetic changes was investigated. In a discovery sample of 93 male Dutch servicemen (PRISMO cohort; same subjects as [17]), specific DNA methylation alterations were associated with the development of PTSD. This cohort displayed changes at 17 positions and 12 regions and subsequent bioinformatic analyses highlighted the role of different pathways linked to PTSD symptomatology. The associations between the development of PTSD symptoms and decreased DNA methylation at genomic regions in *ZFP7*, *RNF39* and *HIST1H2APS2* were replicated in a male US Marine cohort of MRS with a seven-month war-zone deployment to Iraq or Afghanistan (N=98).

Of note, international efforts such as the Psychiatric Genomics Consortium (PGC) PTSD epigenetics group, which combines data from several international military and civilian cohorts, might have increased statistical power to detect further relevant epigenetic variations and thereby provide deeper insights in the near future [38].

Blood Markers

Inflammatory markers

Upon the observation that PTSD co-occurred with peripheral inflammation in cross-sectional studies, the question arose as to whether inflammation markers are causally involved in the disorder [39]. Since then, several prospective studies have attempted to evaluate the causal role of inflammatory responses in the development of PTSD.

One study made use of a subset of the MRS dataset (N=1,719) and found that in U.S. Marines, baseline plasma levels of C-reactive protein (CRP) were a strong predictor of post-deployment PTSD symptoms [23]. Another study analyzed gene co-expression profiles obtained through RNA sequencing of peripheral blood leukocytes from Marines belonging to the MRS II (N=124) and replicated the obtained findings in a separate subsample of the MRS (N=50). The authors found that both at pre- and post-deployment, co-expression gene networks linked to the innate immune responses, interferon signaling and monocyte specificity were predictive of post-deployment PTSD [21]. Using the same cohort, researchers then aimed to build up on these findings and identified several master regulators driving the previously identified networks [22]. Using ARACNe (Algorithm for Reconstruction of Accurate Cellular Networks) and protein activity analysis they identified *SOX3, TNFAIP3, TRAFD1, POU3F3, STAT2*, and *PML* as important master regulators. Gene Ontology analyses enriched for *TNFAIP3, TRAFD1* and *PML* again pointed towards the role of innate immune responses in the development of PTSD.

In a subsample of the PRISMO dataset (N=693), researchers addressed immune activation by measuring *in vitro* cytokine production by leukocytes upon stimulation [19]. The authors observed a three-way interaction between cytokine production one month post-deployment, trauma exposure during combat, and post-deployment stressful life events on changes in PTSD symptoms between 1 month and 2 years post deployment. More specifically, increased mitogen-stimulated T-cell and innate cytokine production, higher stress levels during combat and during the 12-month post-deployment period were associated with increased PTSD symptoms between 1 month and 2 years post-deployment.

Hormonal dysregulations

Another line of studies focused on the hypothalamic–pituitary–adrenal (HPA) axis. Three studies investigated the effects of cortisol levels and stress exposure on PTSD development. Two of these studies were part of the PID-PTSD^{+3} project and assessed hair cortisol concentration (HCC) and cortisol stress reactivity (measured through saliva before and after the Trier Social Stress Test (TSST)), prior to and following a military deployment period of approximately five months [24, 25]. Their main finding showed that when exposed to trauma, a lower baseline HCC and lower cortisol stress level were predictive of higher post-deployment PTSD symptomatology [24] while lower HCC predicted higher daily alcohol consumption [25]. In another study performed in the PRISMO cohort (N=455), plasma cortisol levels at baseline did not predict PTSD status six months after a four months deployment period [18]. Next to cortisol, these researchers further investigated other crucial molecules of the HPA axis. Van Zuiden et al. [18] evaluated the predictive role of mRNA expression of GR–*α*, GR-P, GR-*β*, glucocorticoid-induced leucine zipper (*GILZ*), glucocorticoid-inducible kinase-1 (*SGK-1*), or *FKBP5* in peripheral blood mononuclear cells (PBMCs) and the number of GRs in PBMCs on post-deployment PTSD status. Only the number of GRs in PBMCs predicted post-deployment PTSD status [18].

Another study assessed whether plasma oxytocin (pOT) and arginine vasopressin (pAVP) levels could be used as biomarkers for stress-related development of PTSD [20] in PRISMO. By investigating a group of 907 military subjects, no effects of pOT and pAVP on post-deployment PTSD were observed [20].

Together, these studies highlight the value of prospective studies in linking circulating markers with the development of PTSD. While the first lines of evidence suggests that components of the immune system might emerge as candidate biomarkers, there is apparent need for replication and larger longitudinal studies to confirm and extend these initial findings.

Table 2. Longitudinal human studies assessing biological outcomes associated with differential susceptibility to stress.

Sample (N)	Main stressor	Outcome of interest	Main findings
Genetic factors			
RINGS, Male soldiers: N=253	Deployment	PTSD	Met/Met and Val/Val genotypes had stronger trauma-responses than the Val/Met genotype [29]
Israeli Defense Force, Male soldiers: N=1085	Deployment	PTSD	Threat bias interacted with combat exposure and 5-HTTLPR [26]
Epigenetic factors			
PRISMO and MRS, Male soldiers/marines: N=93, N=98	Deployment	PTSD	Genome-wide methylation changes at 17 positions and 12 regions were associated with PTSD status [16]
PRISMO, Male soldiers: N=92	Deployment	Mental health and PTSD	Pre-deployment GR-1F region (52 loci) methylation did not predict mental health or PTSD status [17]
Circulating markers			
Inflammatory markers			
MRS II and MRS, Male marines: N=124 and 50	Deployment	PTSD	PTSD status associated with gene co-expression networks related to innate immune responses [21]
MRS, Male marines: N=1719	Deployment	PTSD	Baseline plasma levels of CRP predicted PTSD symptoms [23]
PRISMO, N=693	Deployment	PTSD	Interaction between cytokine production, stress exposure during combat and post-deployment stressful life events [19]
MRS II and MRS, Male marines: N=124, N=50	Deployment	PTSD	PTSD status associated with gene co-expression network master regulators *SOX3*, *TNFAIP3*, *TRAFD1*, *POU3F3*, *STAT2*, and *PML* [22]
Hormonal dysregulations			
PRISMO, Male soldiers: N=907	Deployment	PTSD	No moderating effect of plasma oxytocin and arginine vasopressin on stress-related PTSD development [20]
PID-PTSD+3, Male soldiers N=90; N=80	Deployment	PTSD	Decreased baseline hair cortisol and cortisol stress predicted higher stress-related PTSD [24]
PID-PTSD+3, Male soldiers N=153, N=145	Deployment	Alcohol use	Decreased baseline hair cortisol stress predicted higher stress-related alcohol use [25]
PRISMO, Male soldiers: N=68	Deployment	PTSD	Plasma cortisol did not predict PTSD status [18]

MRS, MRS II: Marine Resiliency Study I, II, PID-PTSD^{+3}: Incidence and Determinants of PTSD and Other Mental Disorders, PRISMO: Prospective Research In Stress-Related Military Operations, RINGS: The Readiness and Resilience in National Guard Soldiers Study, CRP: C-reactive protein. Within each section, studies are listed in alphabetical order based on the surname of the first author.

Promoting resilience

The previous section provided an overview of prospective animal and human studies which aimed to gain knowledge of the mechanisms underlying mental illness and resilience. Research in this field has also turned towards studying strategies that promote mental health and boost resilience. As postulated by McEwen et al. [40], the notion that the brain holds the ability to successfully adapt to changing environments throughout the life course, encourages one to develop top-down interventions encompassing mind-body interactions in order to install fundamental changes in various aspects of one's sense of well-being. Given the previously mentioned need to develop strategies to promote resilience in today's society, this section will cover a range of psychological, behavioral and lifestyle interventions aimed at doing so. These, in turn, could be greatly beneficial to individuals with at-risk jobs such as military members who are about to be deployed or have just returned from deployment.

Mindfulness and meditation

Over the last few decades, meditation techniques such as loving-kindness meditation and mindfulness training have become increasingly popular in the western world. Today, mindfulness mainly owes its popularity to Dr. Jon Kabat-Zinn who reintroduced it in his mindfulness-based stress reduction (MBSR) program. Described as the awareness that arises through paying purposeful and non-judgemental attention to the present moment [41], mindfulness is now employed as part of standardized programs aiming to promote general human well-being and cultivate presence.

Empirical evidence about the benefits of mindfulness-based programs are inciting a growing interest in unraveling the (neuro)biological underpinnings of mindfulness. Different mindfulness programs have shown to impact both grey and white matter density of several brain structures such as in the right basolateral amygdala [42] and bilateral clusters within the brainstem including the pontine tegmentum, locus coeruleus, nucleus raphe and the sensory trigeminal nucleus [43]. Moreover, findings show that other types of mind-body interventions also influence the immune system. For example, one study showed that following a yogic meditation, the activity of the proinflammatory nuclear factor-kappa beta (NF-κB), known to have a prominent role in inflammation and stress, was reduced in peripheral blood leukocytes as compared to baseline measures [44]. Another study found an increase in telomerase activity along with reduced levels of inflammatory marker CRP in PBMCs following the same type of meditation [45]. Yet

another study found that by measuring gene expression in peripheral blood prior to and following a deep relaxation session, the expression of genes associated with telomere maintenance were enhanced at follow-up while specific genes linked to stress-related pathways were reduced in expression [46]. Although interesting, it is important to note that currently performed studies vary greatly in the type of (mindfulness) meditation used along with the research designs and sample sizes, which are often (very) small. Gaining knowledge on the mechanisms underlying the well-documented stress-reducing and mood-enhancing effects of meditation and mindfulness-based programs [47-50], holds the potential to further benefit the development of powerful strategies in healthcare settings to promote the cultivation of a healthy mind.

Cognitive behavioral therapy-based programs

Cognitive behavioral therapy (CBT) was originally developed by Aaron Beck to promote mental wellness and coping resources in patients suffering from mental distress such as depression, anxiety and chronic stress [51]. The goal of CBT is to modify one's thinking and behavioral patterns, which color the way life events are being experienced. Interestingly, combining CBT with pharmacological treatments such as cognitive enhancers (but not anxiolytics), has been shown to enhance long-term treatment efficacy and improve fear extinction, potentially by enhancing memory consolidation (recently extensively reviewed in [52]). In addition, conducting CBT sessions such as exposure therapy before sleep has been suggested to enhance treatment efficacy, raising the question whether pharmacological approaches can be implemented to enhance memory consolidations during sleep specifically [52, 53]. Another cognitive intervention targeting memory consolidation involves playing a computer game with high visuospatial demands (e.g. Tetris) during the hours following a traumatic event. This approach has recently been suggested to disrupt the consolidation of traumatic memories and lead to fewer intrusive visual memories of the traumatic event [54]. In order to maximize treatment outcome, combinations of different behavioral approaches with or without pharmacological treatments will need to be tested on an individual level.

Besides its well-documented therapeutic effect in treating mental disorders, today's interest in CBT is also geared towards the construction of a personal model to boost resilience in the face of life's obstacles without necessarily targeting a particular mental disorder. Padesky and Mooney (2012) have proposed a CBT-based program entirely oriented toward 'resiliency' research [55]. Their so-called strengths-based CBT consists of four sessions in which the therapist and the client actively collaborate to explore and

reinforce positive qualities such as interpersonal competences, self-efficacy or self-esteem. The therapist and client co-create a personal model of resiliency by turning the previously identified strengths into effective strategies that can be applied in everyday situations. Another CBT-based program is the Stress Inoculation Therapy (SIT) which was first introduced in 1985 [56]. SIT is a psychotherapeutic intervention that combines cognitive and behavioral methods emphasizing coping skills learning. During a SIT session, an individual is exposed to and learns to cope with increasing amounts of stress through productive thoughts, mental images, self-statements and relaxation training thereby enhancing his or her immunity to stress. Empirical evidence shows that SIT efficiently reduces stress, anxiety and depression in cancer patients [57] and effectively reduces psychological distress up to three months following the sessions when delivered through two half-day training sessions in the workplace [58].

Physical activity

It is commonly known that regularly practicing physical exercises leads to a plethora of positive health effects [59]. These benefits not only include cardiovascular and metabolic effects, but also improvements in cognitive abilities. Previous studies on animals and humans revealed increases in synaptic plasticity and neurogenesis [60], strengthened cortical activation when performing a cognitively challenging task [61] and improvements in learning, slowing the course of cognitive decline in aging [61, 62]. Interestingly, one study showed a causal effect of physical activity on positive affect [63]. Since the extent of positive affect levels varied between individuals, such findings call for individually tailored interventions in which clinicians could adapt the amount of physical exercise to personal needs. Other studies showed that compared to training exercises with no cognitive component, specific exercises that promote mindfulness by means of calming techniques and cognitive strategies such as yoga or pilates were more effective in eliciting psychological benefits such as mood enhancements and improved executive functions over time [64-66]. Mindful-based physical activities seem to improve breathing rate and depth [67, 68] along with heart rate [68] while lowering arousal [69]. Further comparative trials with populations at high risk of being exposed to traumatic stress are needed in order to prospectively assess the effects of these interventions on trauma-related mental illness.

Social support

Several lines of evidence confirm the importance of pursuing cognitive and social activities to maintain global mental and functional health [70-72]. However, the exact

biopsychological mechanisms underlying the positive impact of social support on mental wellbeing and resilience to stress still remain unclear [73]. To enhance both cognitive and social aspects, programs such as the Experience Corps have been introduced. This intergenerational program was originally designed by Fried *et al.* in 1997 to promote health among the aging population. Specifically, this program encourages adults over the age of 50 to share their skills with children needing help at school. While students obtain greater academic outcomes, adults get the opportunity to enrich their lives on social and cognitive levels [74]. A recent study shows that this program further significantly slows the normal age-related decrease in cortical and hippocampal brain volumes [75].

Discussion

As reflected by the present review, there is considerable variation in the way resilience is currently being understood, defined and measured both within animal studies and human studies. Although only a few studies incorporated a baseline behavioral or physiological measure in order to assess dynamic changes over the course of a stressful experience, information gained from these types of studies could yield valuable insights into the molecular mechanisms of differential susceptibility to stress. This, in turn, could lead to the development of potential therapeutic interventions targeting biological "resilience" pathways or to the identification of predictive biomarkers of resilience.

In humans, most of the studies were conducted in military cohorts. While such samples provide a unique opportunity to study the effects of trauma exposure, they are also subjected to a natural limitation since sampling bias cannot be excluded. Researchers are encouraged to include a wider range of assessments when aiming to study and discuss resilience in order to obtain a more reliable and objective operationalization. Using several techniques, e.g. experience-sampling methods [76], in-person interviews combined with self-evaluations, targeted questionnaires and physiological measures including heart rate and blood pressure, will allow one to obtain a more global picture of psychological and physiological health. When possible, this, again, should be embedded within large-scale longitudinal studies to track the stability of one's mental health over a specific time period. Moreover, in order to facilitate extrapolation to the general population, there is a strong need for the inclusion of women in these studies, which is currently underdone.

Finally, alternative strategies aiming to install positive emotions and improve cognitive abilities, social interactions and/or physical health, have obtained scientific evidence for their benefits in increasing one's global mental health. Gaining a deeper knowledge of the positive effects of such interventions, could lead to effective strategies aimed at boosting resilience in more vulnerable individuals.

References

1. Wittchen, H.U., et al., *The size and burden of mental disorders and other disorders of the brain in Europe 2010*. Eur Neuropsychopharmacol, 2011. **21**(9): p. 655-79.
2. Steinert, C., et al., *Relapse rates after psychotherapy for depression - stable long-term effects? A meta-analysis.* J Affect Disord, 2014. **168**: p. 107-18.
3. Kalisch, R., et al., *The resilience framework as a strategy to combat stress-related disorders*. Nature Human Behaviour, 2017. **1**(11): p. 784.
4. Rutten, B.P., et al., *Resilience in mental health: linking psychological and neurobiological perspectives*. Acta Psychiatr Scand, 2013. **128**(1): p. 3-20.
5. Rasmussen, D.D.C., Norman J; Burke, Brianna L, *Acoustic startle amplitude predicts vulnerability to develop post-traumatic stress hyper-responsivity and associated plasma corticosterone changes in rats*. Psychoneuroendocrinology, 2008. **33**(3): p. 282-291.
6. Kim, J.G., et al., *Basal blood corticosterone level is correlated with susceptibility to chronic restraint stress in mice*. Neurosci Lett, 2013. **555**: p. 137-42.
7. Magalhaes, R., et al., *The dynamics of stress: a longitudinal MRI study of rat brain structure and connectome*. Mol Psychiatry, 2017.
8. Chen, R.J., et al., *MicroRNAs as biomarkers of resilience or vulnerability to stress*. Neuroscience, 2015. **305**: p. 36-48.
9. Macfarlane, L.A. and P.R. Murphy, *MicroRNA: Biogenesis, Function and Role in Cancer*. Curr Genomics, 2010. **11**(7): p. 537-61.
10. Tse, Y.C., et al., *A longitudinal study of stress-induced hippocampal volume changes in mice that are susceptible or resilient to chronic social defeat*. Hippocampus, 2014. **24**(9): p. 1120-8.
11. Van Rooij, S., et al., *Smaller hippocampal volume as a vulnerability factor for the persistence of post-traumatic stress disorder*. Psychological medicine, 2015. **45**(13): p. 2737-2746.
12. Kennis, M., et al., *Functional network topology associated with posttraumatic stress disorder in veterans*. NeuroImage: Clinical, 2016. **10**: p. 302-309.
13. Van Rooij, S.J., et al., *Predicting treatment outcome in PTSD: a longitudinal functional MRI study on trauma-unrelated emotional processing*. Neuropsychopharmacology, 2016. **41**(4): p. 1156.
14. van Wingen, G.A., et al., *Persistent and reversible consequences of combat stress on the mesofrontal circuit and cognition*. Proceedings of the National Academy of Sciences, 2012. **109**(38): p. 15508-15513.
15. Hodes, G.E., et al., *Individual differences in the peripheral immune system promote resilience versus susceptibility to social stress*. Proc Natl Acad Sci U S A, 2014. **111**(45): p. 16136-41.
16. Rutten, B.P.F., et al., *Longitudinal analyses of the DNA methylome in deployed military servicemen identify susceptibility loci for post-traumatic stress disorder*. Mol Psychiatry, 2017.
17. Schur, R.R., et al., *Longitudinal changes in glucocorticoid receptor exon 1F methylation and psychopathology after military deployment*. Transl Psychiatry, 2017. **7**(7): p. e1181.

18. van Zuiden, M., et al., *Pre-existing high glucocorticoid receptor number predicting development of posttraumatic stress symptoms after military deployment.* Am J Psychiatry, 2011. **168**(1): p. 89-96.
19. Smid, G.E., et al., *Cytokine production as a putative biological mechanism underlying stress sensitization in high combat exposed soldiers.* Psychoneuroendocrinology, 2015. **51**: p. 534-546.
20. Reijnen, A., E. Geuze, and E. Vermetten, *Individual variation in plasma oxytocin and vasopressin levels in relation to the development of combat-related PTSD in a large military cohort.* J Psychiatr Res, 2017. **94**: p. 88-95.
21. Breen, M.S., et al., *Gene networks specific for innate immunity define post-traumatic stress disorder.* Mol Psychiatry, 2015. **20**(12): p. 1538-45.
22. Torshizi, A.D. and K. Wang, *Deconvolution of Transcriptional Networks in Post-Traumatic Stress Disorder Uncovers Master Regulators Driving Innate Immune System Function.* Scientific reports, 2017. **7**(1): p. 14486.
23. Eraly, S.A., et al., *Assessment of plasma C-reactive protein as a biomarker of posttraumatic stress disorder risk.* JAMA psychiatry, 2014. **71**(4): p. 423-431.
24. Steudte-Schmiedgen, S., et al., *Hair cortisol concentrations and cortisol stress reactivity predict PTSD symptom increase after trauma exposure during military deployment.* Psychoneuroendocrinology, 2015. **59**: p. 123-33.
25. Trautmann, S., et al., *Biological stress indicators as risk markers for increased alcohol use following traumatic experiences.* Addict Biol, 2018. **23**(1): p. 281-290.
26. Wald, I., et al., *Attention to threats and combat-related posttraumatic stress symptoms: prospective associations and moderation by the serotonin transporter gene.* JAMA Psychiatry, 2013. **70**(4): p. 401-8.
27. Hoge, C.W., et al., *Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care.* New England Journal of Medicine, 2004. **351**(1): p. 13-22.
28. American Psychiatric Association, *Diagnostic and statistical manual of mental disorders (DSM-5®)*. 2013: American Psychiatric Pub.
29. Clark, R., et al., *Predicting post-traumatic stress disorder in veterans: interaction of traumatic load with COMT gene variation.* J Psychiatr Res, 2013. **47**(12): p. 1849-56.
30. King, L.A., et al., *Deployment Risk and Resilience Inventory: A collection of measures for studying deployment-related experiences of military personnel and veterans.* Military Psychology, 2006. **18**(2): p. 89-120.
31. Zuj, D.V., et al., *The centrality of fear extinction in linking risk factors to PTSD: A narrative review.* Neurosci Biobehav Rev, 2016. **69**: p. 15-35.
32. Kolassa, I.-T., et al., *The risk of posttraumatic stress disorder after trauma depends on traumatic load and the catechol-O-methyltransferase Val158Met polymorphism.* Biological psychiatry, 2010. **67**(4): p. 304-308.
33. Hayes, J.P., et al., *COMT Val158Met polymorphism moderates the association between PTSD symptom severity and hippocampal volume.* Journal of psychiatry & neuroscience: JPN, 2017. **42**(2): p. 95.
34. Zannas, A.S., N. Provencal, and E.B. Binder, *Epigenetics of Posttraumatic Stress Disorder: Current Evidence, Challenges, and Future Directions.*

Biol Psychiatry, 2015. **78**(5): p. 327-35.
35. Vinkers, C.H., et al., *Traumatic stress and human DNA methylation: a critical review.* Epigenomics, 2015. **7**(4): p. 593-608.
36. Mehta, D., et al., *Childhood maltreatment is associated with distinct genomic and epigenetic profiles in posttraumatic stress disorder.* Proceedings of the national academy of sciences, 2013. **110**(20): p. 8302-8307.
37. Uddin, M., et al., *Gene expression and methylation signatures of MAN2C1 are associated with PTSD.* Dis Markers, 2011. **30**(2-3): p. 111-21.
38. Ratanatharathorn, A., et al., *Epigenome-wide association of PTSD from heterogeneous cohorts with a common multi-site analysis pipeline.* Am J Med Genet B Neuropsychiatr Genet, 2017. **174**(6): p. 619-630.
39. Baker, D.G., C.M. Nievergelt, and D.T. O'Connor, *Biomarkers of PTSD: neuropeptides and immune signaling.* Neuropharmacology, 2012. **62**(2): p. 663-73.
40. McEwen, B.S., J.D. Gray, and C. Nasca, *Redefining neuroendocrinology: stress, sex and cognitive and emotional regulation.* The Journal of endocrinology, 2015. **226**(2): p. T67-T83.
41. Kabat-Zinn, J., *Mindfulness-Based Interventions in Context: Past, Present, and Future.* Clinical Psychology: Science and Practice, 2006. **10**(2): p. 144-156.
42. Hölzel, B.K., et al., *Stress reduction correlates with structural changes in the amygdala.* Social Cognitive and Affective Neuroscience, 2010. **5**(1): p. 11-17.
43. Singleton, O., et al., *Change in Brainstem Gray Matter Concentration Following a Mindfulness-Based Intervention is Correlated with Improvement in Psychological Well-Being.* Front Hum Neurosci, 2014. **8**: p. 33.
44. Black, D.S., et al., *Yogic meditation reverses NF-kappaB and IRF-related transcriptome dynamics in leukocytes of family dementia caregivers in a randomized controlled trial.* Psychoneuroendocrinology, 2013. **38**(3): p. 348-55.
45. Lavretsky, H., et al., *A pilot study of yogic meditation for family dementia caregivers with depressive symptoms: effects on mental health, cognition, and telomerase activity.* Int J Geriatr Psychiatry, 2013. **28**(1): p. 57-65.
46. Bhasin, M.K., et al., *Relaxation response induces temporal transcriptome changes in energy metabolism, insulin secretion and inflammatory pathways.* PLoS One, 2013. **8**(5): p. e62817.
47. Tang, Y.Y., et al., *Short-term meditation training improves attention and self-regulation.* Proc Natl Acad Sci U S A, 2007. **104**(43): p. 17152-6.
48. Goleman, D.J. and G.E. Schwartz, *Meditation as an intervention in stress reactivity.* J Consult Clin Psychol, 1976. **44**(3): p. 456-66.
49. Ding, X., et al., *Improving creativity performance by short-term meditation.* Behav Brain Funct, 2014. **10**: p. 9.
50. Jain, S., et al., *A randomized controlled trial of mindfulness meditation versus relaxation training: effects on distress, positive states of mind, rumination, and distraction.* Ann Behav Med, 2007. **33**(1): p. 11-21.
51. Beck, A.T., *Cognitive therapy and the emotional disorders*. 1976, New York: International Universities Press.
52. Singewald, N., et al., *Pharmacology of cognitive enhancers for exposure-based therapy of fear, anxiety and trauma-related disorders.*

Pharmacology & therapeutics, 2015. **149**: p. 150-190.
53. Pace-Schott, E.F., et al., *Sleep promotes consolidation and generalization of extinction learning in simulated exposure therapy for spider fear*. Journal of psychiatric research, 2012. **46**(8): p. 1036-1044.
54. Iyadurai, L., et al., *Preventing intrusive memories after trauma via a brief intervention involving Tetris computer game play in the emergency department: a proof-of-concept randomized controlled trial.* Molecular psychiatry, 2018. **23**(3): p. 674.
55. Padesky, C.A. and K.A. Mooney, *Strengths-based cognitive-behavioural therapy: a four-step model to build resilience.* Clin Psychol Psychother, 2012. **19**(4): p. 283-90.
56. Meichenbaum, D., *Stress inoculation training*. 1985, Elmsford, NY: Pergamon Press.
57. Kashani, F., et al., *Effect of stress inoculation training on the levels of stress, anxiety, and depression in cancer patients.* Iran J Nurs Midwifery Res, 2015. **20**(3): p. 359-64.
58. Flaxman, P.E. and F.W. Bond, *A randomised worksite comparison of acceptance and commitment therapy and stress inoculation training.* Behav Res Ther, 2010. **48**(8): p. 816-20.
59. Penedo, F.J. and J.R. Dahn, *Exercise and well-being: a review of mental and physical health benefits associated with physical activity.* Curr Opin Psychiatry, 2005. **18**(2): p. 189-93.
60. van Praag, H., et al., *Running enhances neurogenesis, learning, and long-term potentiation in mice.* Proc Natl Acad Sci U S A, 1999. **96**(23): p. 13427-31.
61. Colcombe, S.J., et al., *Cardiovascular fitness, cortical plasticity, and aging.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3316-21.
62. Erickson, K.I., et al., *Exercise training increases size of hippocampus and improves memory.* Proc Natl Acad Sci U S A, 2011. **108**(7): p. 3017-22.
63. Wichers, M., et al., *A time-lagged momentary assessment study on daily life physical activity and affect.* Health Psychol, 2012. **31**(2): p. 135-44.
64. Manjunath, N.K. and S. Telles, *Improved performance in the Tower of London test following yoga.* Indian J Physiol Pharmacol, 2001. **45**(3): p. 351-4.
65. Dale, L.P., et al., *Yoga Workshop Impacts Psychological Functioning and Mood of Women With Self-Reported History of Eating Disorders.* Eating Disorders, 2009. **17**(5): p. 422-434.
66. Netz, Y. and R. Lidor, *Mood alterations in mindful versus aerobic exercise modes.* J Psychol, 2003. **137**(5): p. 405-19.
67. Robert McComb, J.J., et al., *A pilot study to examine the effects of a mindfulness-based stress-reduction and relaxation program on levels of stress hormones, physical functioning, and submaximal exercise responses.* J Altern Complement Med, 2004. **10**(5): p. 819-27.
68. Danucalov, M.A., et al., *Cardiorespiratory and metabolic changes during yoga sessions: the effects of respiratory exercises and meditation practices.* Appl Psychophysiol Biofeedback, 2008. **33**(2): p. 77-81.
69. Telles, S., S.K. Reddy, and H.R. Nagendra, *Oxygen consumption and respiration following two yoga relaxation techniques.* Appl Psychophysiol Biofeedback, 2000. **25**(4): p. 221-7.

70. Fratiglioni, L., S. Paillard-Borg, and B. Winblad, *An active and socially integrated lifestyle in late life might protect against dementia.* The Lancet Neurology, 2004. **3**(6): p. 343-353.
71. Cohen, S., *Social relationships and health.* Am Psychol, 2004. **59**(8): p. 676-684.
72. Lorenz, R.A., et al., *Exercise and social activity improve everyday function in long-term care residents.* Am J Geriatr Psychiatry, 2012. **20**(6): p. 468-76.
73. Ozbay, F., et al., *Social support and resilience to stress: from neurobiology to clinical practice.* Psychiatry (Edgmont), 2007. **4**(5): p. 35.
74. Fried, L.P., et al., *A social model for health promotion for an aging population: initial evidence on the Experience Corps model.* J Urban Health, 2004. **81**(1): p. 64-78.
75. Carlson, M.C., et al., *Impact of the Baltimore Experience Corps Trial on cortical and hippocampal volumes.* Alzheimer's & dementia: the journal of the Alzheimer's Association, 2015. **11**(11): p. 1340-1348.
76. Verhagen, S.J., et al., *Use of the experience sampling method in the context of clinical trials.* Evidence-based mental health, 2016. **19**(3): p. 86-89.

CHAPTER 3

MicroRNAs in post-traumatic stress disorder

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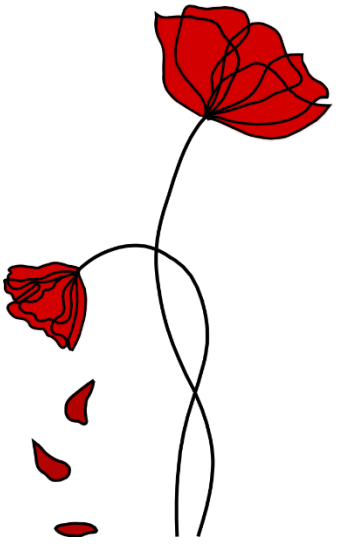

Abstract

Post-traumatic stress disorder (PTSD) is a psychiatric disorder that can develop following exposure to, or witnessing a (potentially) traumatic event. A critical issue is to pinpoint (neuro)biological mechanisms underlying susceptibility to stress-related disorders such as PTSD. Over the last few years, a first wave of epigenetic studies has attempted to identify molecular underpinnings of the long-lasting effects of trauma exposure. The potential roles of non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) in regulating the impact of severe stress and trauma are increasingly gaining attention. The present review offers an overview of studies of miRNAs in PTSD and discusses the current challenges, pitfalls and future perspectives of the field. Most studies have been completed in animals, use cross-sectional study designs and focus on subjects with susceptible phenotypes only. There is a great need for future research to comprise translational and cross-species approaches that use longitudinal designs to study trajectories of change contrasting susceptible and resilient subjects.

Keywords: epigenetics, microRNAs, post-traumatic stress disorder, brain, review

Introduction

Over the last few decades, epigenetic mechanisms have been proposed to be key mediators of the lasting behavioral and molecular effects of traumatic stress exposure [1]. While the first epigenetic studies in this area focused mostly on DNA methylation, epigenetic studies in more recent years have expanded this approach by analyzing the expression of non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and their impact on gene expression. miRNAs are increasingly being investigated for their pathophysiological connection to psychiatric disorders including post-traumatic stress disorder (PTSD). More recently, studies have started to focus on the potential use of miRNAs as biomarkers of PTSD.

The present review provides an overview of the available literature on miRNAs in relation to traumatic stress and their impact on mental health in humans and rodents. First, we briefly describe PTSD-related neurobiological alterations along with the basic concepts of epigenetic mechanisms. Next, an overview of the current scientific evidence on miRNAs in relation to PTSD in humans and PTSD-related symptoms in animals is provided. Finally, current challenges, pitfalls and future perspectives in studying the potential roles of miRNAs in PTSD are discussed.

Post-traumatic stress disorder

PTSD is a psychiatric disorder that is triggered by a (potentially) life-threatening traumatic event, i.e., an event capable of producing intense feelings of fear, helplessness and horror [2]. Characteristic symptoms include re-experiencing of the traumatic event through intrusive imagery or recurrent nightmares, avoidance of reminders to the event, negative mood, and hyperarousal reflected by insomnia and/or hypervigilance. Although these symptoms are often of limited intensity and duration, in a small portion of the population they persist longer than one month following trauma exposure and create significant distress. Long-term persistence of symptoms is characteristic of PTSD, while the ability to withstand trauma and rapidly recover from an acute stress reaction without progression to PTSD, is referred to as resiliency.

Over the past few decades, PTSD has repeatedly been associated with several neurobiological alterations including decreased hippocampal volume [3-5], hyperactivity of the amygdala and hypoactivity of the dorsal and rostral anterior cingulate cortex (ACC) and ventromedial prefrontal cortex (vmPFC) [5-7]. In an attempt to further elucidate the

(neuro)biological processes underlying the observed differential susceptibility to traumatic stress, a large number of studies have focused on alterations in the hypothalamus-pituitary-adrenal (HPA) axis. Since the HPA axis is a core component of the mammalian stress response, its (dys)function has been extensively studied in the context of PTSD. In healthy individuals, stressful events trigger neurons of the hypothalamic paraventricular nucleus (PVN) to secrete corticotropin-releasing hormone (CRH) and vasopressin, which causes the release of adrenocorticotropin (ACTH) from the anterior pituitary and finally glucocorticoids from the adrenal cortex [8]. The activity of the HPA axis is modulated via several brain regions; for example, neurons of the PVN are inhibited by the hippocampus and PFC and stimulated by areas such as the amygdala [9]. Additionally, in order to regulate their own synthesis, glucocorticoids inhibit excessive synthesis and release of CRH and ACTH by controlling hippocampal and PVN neurons, and downregulating CRH_1 receptors and corticotrope function in the anterior pituitary, thereby creating a negative feedback mechanism [10].

Several studies have found that individuals with PTSD show increased levels of CRH in their cerebrospinal fluid (CSF) [11], as well as a blunted ACTH response to CRH [12], a disturbed negative feedback loop [13], increased sensitivity of glucocorticoid receptors (GRs) and chronically lowered cortisol levels [14, 15]. Although dysregulation of the HPA axis is well-documented in stress-related disorders and PTSD has repeatedly been associated with reduced cortisol levels, individual variability remains. The current hypothesis is that cortisol levels depend upon gender and the type of trauma exposure, among other factors [16-18]. To further unravel the molecular regulation of biological mechanisms underlying the onset and course of PTSD, more recent research has focused on the involvement of epigenetic mechanisms.

Epigenetics: the roles of microRNAs

The term epigenetics refers to a variety of environmentally-triggered, heritable but reversible processes involved in the regulation of gene expression without alteration of the original genetic code [19]. These epigenetic modifications are numerous and include (hydroxy)methylation of DNA cytosine residues, post-translational modifications (PTMs) of histone proteins and ncRNAs [20, 21]. ncRNAs refer to a class of small RNA molecules that are transcribed from genomic DNA without being translated into proteins [19]. Instead, these RNAs are directly involved in cellular function and gene expression regulation. Next to ribosomal and transfer RNAs, ncRNAs include the more commonly

studied small interfering RNAs [22], circular RNAs [23], piwi-interacting RNAs [24], and miRNAs.

Biogenesis and mode of action of microRNAs

miRNAs are small (~ 22nt in length) ncRNA molecules found in most eukaryotes [25]. Thousands of different miRNAs are expressed within an organism and are involved in post-transcriptional regulation of gene expression [26]. miRNAs are commonly classified as intergenic or intronic. While intergenic miRNA are transcribed from stretches of noncoding DNA by RNA polymerase II and/or III [27], intronic miRNAs are processed mostly from gene introns [28]. In both cases, a primary miRNA (pri-miRNA) is formed and further cleaved and stabilized by the protein complex microprocessor that includes the ribonuclease III Drosha and its co-factor, DiGeorge syndrome critical region 8 (DGCR8) [27]. This process takes place within the nucleus and results in a precursor miRNA (pre-miRNA) of 70-100nt forming a hairpin structure [29, 30]. Following transport to the cytoplasm by the nuclear transport factor Exportin-5, a complex including the RNase III Dicer further processes the pre-miRNA to yield a miRNA duplex containing the final mature miRNA strand and a so-called passenger strand (Figure 1) [31].

Currently, miRNAs are thought to regulate approximately 30 to 60% of human protein-coding genes [32, 33]. Binding of the 5′ end of the mature miRNA (i.e. the 'seed' sequence) to an almost complementary 6-8nt seed match sequence in the 3′ UTR of mRNAs, induces mRNA degradation or translational inhibition [26, 31]. Since base-pair matching between miRNAs and mRNAs relies on imprecise complementarity, one single miRNA can target many different mRNAs. Specifically, mature miRNAs trigger the activation of the RNA-induced silencing complex (RISC), a large protein complex containing an Argonaute protein (Ago2) and the mature single-stranded miRNA that leads the complex towards the appropriate mRNA target [25]. It was commonly believed that, at this point, only the functional guide strand of the double-stranded miRNA product was incorporated into the RISC and the passenger strand was degraded [29]. However, increasing evidence shows that the passenger strand also has biological functions and targets mRNAs [34]. In either case, depending on the type of Ago protein, the target will either be cleaved directly or additional proteins may be needed in order to achieve gene silencing. However, exactly how this complex interacts with mRNA strands and which additional proteins are recruited, remains unclear.

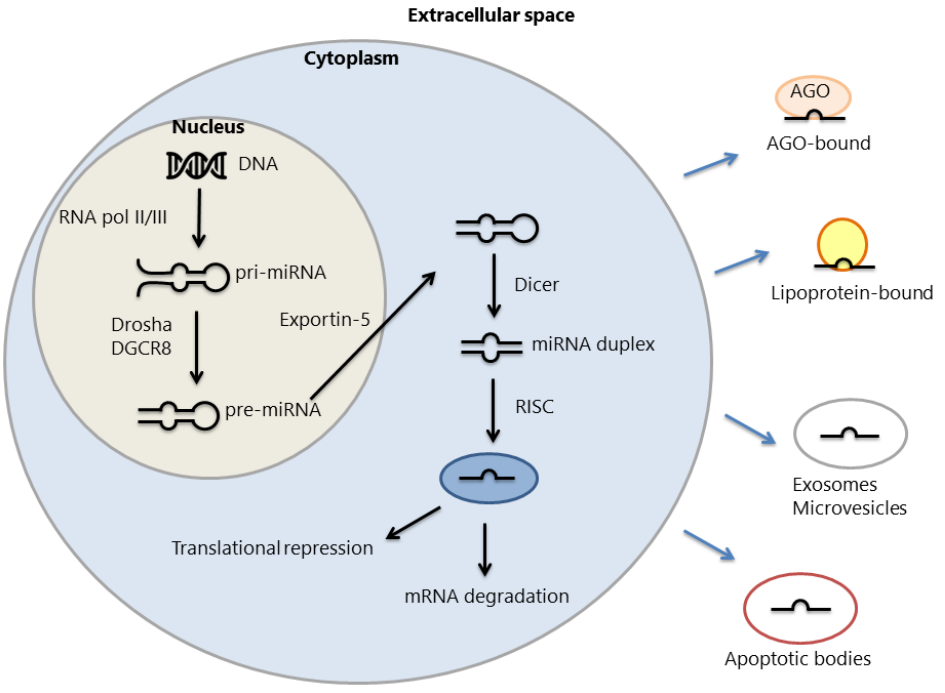

Figure 1. Biogenesis and (extra)cellular locations of miRNAs. miRNAs are transcribed into pri-miRNA by RNA polymerase II and/or III before being further processed by Drosha and DGCR8 to form a cleaved pre-miRNA. After transportation to the cytoplasm by Exportin-5, this fragment is further digested by a complex including the RNase III Dicer. The mature miRNA is then involved in translational repression and/or mRNA degradation through interaction with the RISC. In the extracellular space, miRNAs are protected from degradation by RNases through binding to RNA-binding proteins (e.g. Ago 1 or 2) or (high-density) lipoproteins, or packaging into extracellular vesicles such as exosomes or microvesicles.

Circulating microRNAs

While most miRNAs are found inside cells, a significant number of miRNAs have been observed in extracellular compartments of blood plasma, serum, saliva, urine and CSF [35-38]. These extracellular miRNAs are relatively stable due to being bound to proteins such as Ago1 or 2 and (mostly high density) lipoproteins or packed into vesicles and thus protected from degradation by RNases (Figure 1) [36, 39-44]. Packaging of miRNAs is the most common mechanism used to protect circulating miRNAs. miRNAs can be packaged into apoptotic bodies, shedding vesicles called microvesicles, or exosomes resulting from multivesicular bodies (MVBs) fusing with the plasma membrane [36, 39]. miRNAs encapsulated within MVBs are believed to arise from the disassembled RISC and are packed along with several RISC-associated components [45]. Once secreted, exosomes translocate easily across cell membranes, thus allowing miRNAs to be taken up by other

cells where they hold the potential to actively alter mRNA stability [46]. Although packed miRNAs are thought to be specifically involved in RNA-mediated cell-to-cell communication, Ago-bound miRNAs appear to be non-specific residues of cellular activity or cell death [39]. So far, Ago-miRNA complexes have not been found to be actively released or taken up by recipient cells, unlike exosomal miRNAs [39]. Although several theories have been postulated with regard to the origin, stability and precise function of extracellular miRNAs in recipient cells, many questions remain to be answered. Still, circulating miRNAs have several properties that make them interesting relevant biomarker candidates; they are stable in various biofluids, their sequences are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be relatively easily assessed by various methods, such as small-RNA sequencing, microarrays and quantitative polymerase chain reaction (qPCR) [47].

MicroRNAs in the central nervous system

miRNAs are widely expressed within the central nervous system (CNS) and are suggested to be crucially involved in its development [48]. Studies have demonstrated that several miRNAs are implicated in the proliferation and differentiation of neural stem cells (NSCs) [49], dendritic development [50], axon outgrowth and branching [51] and synaptic plasticity [52, 53]. Given their central involvement in neural development and function, several dysregulated miRNAs within the CNS have been identified in neuropsychiatric and neurodegenerative disorders such as major depressive disorder (MDD) [54, 55], Alzheimer's disease (AD) [56, 57] and Parkinson's disease (PD) [58, 59]. Identifying exactly how miRNAs within the CNS exert their regulatory effects will be crucial for our understanding of their precise involvement in these and other neurological disorders.

MicroRNAs, stress and PTSD

The results of studies examining miRNAs related to PTSD-like symptoms in animals, or stress and PTSD in humans, are described below and summarized in Tables 1 and 2, respectively.

Animal studies

MicroRNAs, fear conditioning and memory formation

Individuals with PTSD are known to show enhanced fear conditioning which can be alleviated through exposure-based therapy [60]. This therapy could be considered as the

human equivalent of using fear extinction training in animals [61]. The first study that indirectly examined the roles of miRNAs in PTSD focused on their involvement in fear extinction [62]. In this study, the levels of miR-128b were found increased in the infra-limbic PFC (ilPFC) of mice following fear extinction training, highlighting its potential involvement in fear conditioning [62]. Previously, proteins involved in miRNA biogenesis had already been shown to play a role in memory formation. For example, the deletion of Dicer1 in the forebrain of mice caused a decrease in several miRNAs and enhanced learning and memory strength [63]. Several animal studies have since confirmed that specific miRNAs in several brain regions are involved in fear memory consolidation [64, 65], contextual fear memory [66], state-dependent fear [67] and memory acquisition of trace fear conditioning [68].

Circulating microRNAs as biomarkers of post-traumatic stress disorder

Over the past few years, fluctuations of miRNA levels in body fluids have been found to correlate with psychiatric disorders, including MDD [69], schizophrenia [70] and bipolar disorder [71]. These studies suggest potential for the use of circulating miRNAs as diagnostic biomarkers of mental disorders. The first study investigating circulating miRNAs as biomarkers of PTSD-related symptoms found that the expression of nine miRNAs was increased both in the amygdala and serum of rats exposed to three days of immobilization and tail shock sessions [72]. One of the increased stress-responsive miRNAs, miR-19b, was also found to be involved in the regulation of fear-associated genes. A third lead for miR-19b involvement comes from a study using mice undergoing chronic social defeat stress (CSDS) and reporting significant increases in the basolateral amygdala (BLA) following CSDS as compared to non-stressed controls [73]

More recently, the potential use of miRNAs as biomarkers of both vulnerability and resilience to stress was examined. In one study, circulating miRNA profiles were examined three days before and 24h following CSDS in rats [74]. Prior to the stressful event, four miRNAs (miR-4-2-5p, miR-27a-3p, miR-30e-5p, miR-362-3p) were significantly decreased only in those rats that later became vulnerable to stress. Following stress exposure, four different miRNAs (miR-139-5p, miR-28-3p, miR-326-3p, miR-99b-5p) were decreased in resilient animals. These results show that different miRNAs potentially confer vulnerability to future stress or promote sustained resilience. Taken together, these studies show preliminary promise for using miRNAs as biomarkers of vulnerability and resiliency to stress.

MicroRNAs in transgenerational inheritance of early stress

Several animal studies have shown that ncRNAs are abundantly present in sperm and may be involved in non-Mendelian inheritance of behavioral phenotypes [75, 76]. Therefore, to assess the potential role of miRNAs in the transgenerational inheritance of parental stress, Gapp *et al.* [77] examined sperm samples of a mouse model of unpredictable maternal separation with unpredictable maternal stress (MSUS). Several miRNAs (among other ncRNAs) were upregulated in F1 MSUS sperm (but not F2 sperm) as compared to the sperm of non-stressed control mice. Several miRNA levels were further altered in the serum, hippocampus and hypothalamus of F1 MSUS mice, and in the serum and hippocampus of F2 MSUS mice. Interestingly, following injection of RNAs purified from MSUS male sperm into wild-type fertilized mouse oocytes, similar behavioral, metabolic and molecular effects were obtained as compared to direct exposure to MSUS. Additionally, the offspring of these mice showed depressive-like behaviors. These and other results [78] provide support for the involvement of specific miRNAs in the transgenerational transmission of behavioral phenotypes.

MicroRNAs targeting the FK506 Binding Protein 5 (FKBP5) gene

Genetic variations in *FKBP5* have been extensively studied in the context of gene x environment (GxE) interactions and the influence of early life adversity on the development of PTSD [79-81]. FKBP5 is a HSP90 co-chaperone that strongly controls glucocorticoid receptor (GR) sensitivity and signaling by binding to GRs in the cytosol thereby decreasing GR ligand affinity and nuclear translocation [82]. Several studies have shown that homozygous genotypes for SNPs in *FKBP5* interact with early life (but not adult) adversity, increasing the risk for later development of PTSD [79, 83]. Epigenetic mechanisms have repeatedly been found to contribute to the regulation of *FKBP5* expression [84, 85]. Moreover, FKBP51, one of the proteins encoded by *FKBP5,* presents an interesting target for the treatment of stress-related disorders. Increased levels of FKBP51 have been suggested to increase the risk of MDD and PTSD and the deletion of *FKBP5* has been shown to prevent age-related depression-like phenotypes [86]. However, pharmacologically targeting FKBP51 has proven to be challenging due to the strong sequence similarity between this and other FKBP proteins [87].

Recently, two independent studies have shown that miR-15a and miR-511 potentially affect FKBP51 levels by targeting FKBP5 mRNAs [88, 89]. In the first study, FKBP51 levels were found decreased and miR-15a levels significantly increased in the amygdala of mice subjected to CSDS as compared to non-stressed controls [89]. This same pattern was found in peripheral blood of healthy humans following dexamethasone treatment and in

individuals exposed to early life trauma [89]. In the second study, FKBP5 mRNA and protein levels were found to be decreased by miR-511, which was further shown to be involved in neuronal differentiation [88]. These findings indicate that both miRNAs could potentially be interesting candidates for the exploration of treatment strategies of stress-related disorders and set the foundations for further studies to examine the exact roles of both miRNAs in *FKBP5* regulation.

Table 1. Animal studies examining miRNAs in PTSD.

Species and sex	**Model**	**Tissue**	**miRNA analyses**	**Main findings**
Male rats	Surgical traumatic stress, cultured neurons	Frontal cortex	TaqMan miRNA assay, qRT-PCR	↑ miR-222 in the frontal cortex 3d following traumatic stress [90]
Male rats	Auditory FC	Amygdala	qRT-PCR, miRNA microarray, TaqMan miRNA assay, miRNA overexpression	↓ miR-182 1h following auditory FC. Overexpression in lateral amygdala disrupted long-term memory formation [65]
Male rats	3d of immobili-zation and tail shock sessions	Serum, amygdala	qRT-PCR, TaqMan miRNA assay	↑ miR-142-5p, miR-19b, miR-1928, miR-223-3p, miR-322*, miR-324, miR-421-3p, miR-463*, miR-674* in serum & amygdala [72]
Male rats	7d CSDS	mPFC, BLA, circulation	MiRNA microarray, qPCR	Vulnerability to stress is associated with: Circulation: ↓ miR-24-2-5p, miR-27a-3p, miR-30e-5p, miR-3590-3p, miR-362-3p, miR-532-5p mPFC: ↑ miR-126a-3p, miR-708-5p. BLA: 77 dysregulated miRNAs, none associated with vulnerability to stress [74]
Male rats	6d of electric FS	Hypotha-lamus	RT-PCR	Traumatic stress was related to ↑ miR-34c in the hypothalamus [91]
Mice	Fear extinction training	ILPFC	Lentiviral vector (miR KD/ overexpression)	miR-128b is involved in formation of fear extinction memory [62]
Male mice	42d of chronic variable stress	Sperm	TaqMan miRNA assay	↑ miR-193*, miR-204, miR-29c, miR-30a, miR-30c, miR-32, miR-375, miR-532-3p, miR-698 in parental sperm [78]

Male mice	Single electric FS	PFC	Microarray, RT-qPCR	FXT administration in shocked mice causes ↓ mmu-miR-1971 expression [92]
Male mice	FC	Hippocam-pus	Lentiviral vector (miR KD), TaqMan miRNA assay	↑ miR-132 30 min after trace FC. Overexpression in hippocampus impairs FC acquisition [68]
Male mice	Social defeat stress	Heart	miRNA array	Heart injury following social stress was associated with decreased miR-29b, miR-302a and let-7d levels in one strain [93]
Male mice	Auditory FC	BLA	miRNA microarray, luciferase assay	miR-34a is involved in fear memory consolidation [64]
Male mice	MSUS	Sperm, serum, brain	Deep sequencing, qRT-PCR	↑ miR-375-3p and -5p, miR-200b-3p, miR-672-5p, miR-466-5p in F1 sperm, serum, hippocampus and hypothalamus and in F2 serum and hippocampus [77]
Mice	FC	Hippocam-pus	Lentiviral vector (miR KD), TaqMan miRNA assay	Inhibition of miR-92 in hippocampus impairs contextual fear conditioning [66]
Male mice	10d CSDS	Amygdala	miRNA microarray, qRT-PCR	miR-19b associates with Ago2, regulates Adrb1, and is significantly elevated in the amygdala of stressed mice [73]
Male mice	Contex-tual FC	Hippocam-pus	miRNA microarray	miR-33 regulates GABA-related proteins [67]
Male and female mice	Cell cultures	Cortex	qPCR, Luciferase assay, mRNA pulldown assay.	miR-511 targets and suppresses FKBP5 mRNA and protein levels [88]
Male mice	10d CSDS	Amygdala	miRNA microarray	miR-15a associates with Ago2, increases following chronic stress, and downregulates FKBP51 levels [89]

Studies are grouped according to species and listed in chronological order within groups. The sex of the animals is missing if it was not mentioned in the paper. FC: fear conditioning, d: days, ctrl: non-stressed controls, qRT-PCR: quantitative reverse transcription polymerase chain reaction, miR: microRNA, CSDS: chronic social defeat stress, mPCF: medial prefrontal cortex, BLA: basolateral amygdala, qPCR: quantitative polymerase chain reaction, ILPFC: infra-limbic prefrontal cortex, KD: knockdown, FS: foot shock, FXT: Fluoxetine, MSUS: unpredictable maternal separation combined with unpredictable maternal stress, mRNA: messenger RNA.

Human studies

Most human studies identified miRNAs related to immunological dysregulations which are well documented in PTSD [94, 95]. PTSD has repeatedly been linked to an excessive inflammatory state, possibly resulting from insufficient counter-regulation due to cortisol hyposecretion [96, 97]. The first study examining peripheral blood mononuclear cells (PBMCs) of combat veterans diagnosed with PTSD found that alterations in specific miRNAs correlated with immunological changes [98]. Specifically, miR-125a and miR-181c were significantly decreased in PTSD patients as compared to healthy controls. Further analyses revealed that miR-125a downregulated the production of the pro-inflammatory cytokine IFN-γ by targeting its gene. This suggests that the observed increase in IFN-γ in PBMCs of PTSD subjects appears to be, at least in part, epigenetically regulated. Intriguingly, miR-27a-3p, which was downregulated in the circulation of rats vulnerable to future stress [74], and miR-19b [72, 73] and miR-223 [72] which were increased in the serum and the amygdala of stressed rodents, were also dysregulated in the present cohort of combat veterans with PTSD [98]. However, while two independent animal studies found increased miR-19b levels in several tissues following stress exposure [72, 73] and one study reported increased levels of miR-223 [72], the same miRNAs were significantly decreased in PBMCs of the human cohort [98].

Following this initial study linking miRNAs and immune dysfunctions in PTSD, two recent studies performed by the same research group provide further evidence for the implication of epigenetic mechanisms in inflammation profiles in PTSD [99, 100]. In addition to IFN-γ, the pro-inflammatory cytokine IL-12 was increased in the same cohort of combat veterans, and miR-193a-5p, which is suggested to target IL-12B, was downregulated [99]. These results provide further evidence that pro-inflammatory gene expression could be regulated by miRNAs.

Recently, one study found that 8 miRNAs were differentially expressed (4 upregulated and 4 downregulated) in peripheral blood samples of returning combat veterans as compared to controls [101]. Pathway analyses revealed that these miRNAs target genes involved in Wnt signaling and axon guidance. However, being limited by a small sample size, this study encourages larger studies to further unravel the involvement of miRNAs in PTSD vulnerability.

Table 2. Human studies examining miRNAs in PTSD.

Population	Sample size	Tissue	miRNA analyses	Main finding
Male and female combat veterans with PTSD	12 (8 PTSD, 22 ctrl)	PBMCs	miRNA microarray, RT-PCR	↓ miR-125a, miR-181c [98]
Male and female patients with PTSD and comorbid depression	78 (51 PTSD&dep, 27 ctrl)	Whole blood	qPCR, RNA-seq	↓ miR-3130-5p, ↓ *DICER1* mRNA levels [102]
Male individuals	26 (healthy)	Whole blood	miRNA microarray	↑ miR-15a following DEX administration or childhood trauma exposure [89]
Male combat veterans with PTSD	33 (16 PTSD, 17 ctrl)	PBMCs	qRT-PCR	↓ miR-193a-5p [99]
Male combat veterans with PTSD	48 (24 PTSD, 24 ctrl)	PBMCs	RNA-seq, miRNA microarray, qRT-PCR	190 differentially expressed miRNAs, 7 ↑, 183 ↓ [100]
Male combat veterans with PTSD	24 (15 PTSD, 9 ctrl)	Whole blood	miRNA-seq	8 differentially expressed miRNAs; 4 ↑, 4 ↓ [101]

Studies are listed in chronological order. DEX: dexamethasone, PBMCs: peripheral blood mononuclear cells, PTSD&dep: PTSD with comorbid depression, (mi)RNA-seq: (micro)RNA-sequencing.

Current challenges, pitfalls, and future perspectives

As reflected by the present overview, most studies that examined the roles of miRNAs in PTSD used (almost exclusively male) animals. Human studies are now beginning to emerge and have so far examined peripheral blood samples only. Moreover, most studies have focused on susceptible phenotypes only, i.e., those animals and individuals suffering the consequences of trauma exposure. To the best of our knowledge, only one study has examined the potential of miRNAs to serve as biomarkers of both vulnerability and resilience [74]. Important limitations of current epigenetic research in PTSD includes the use of small sample sizes and the lack of longitudinal studies that would enable the identification of dynamic epigenetic changes over time. Future research is critically needed to overcome a few pressing issues.

First, given the tissue specificity of epigenetic alterations and the evident inability to study the brains of living human beings, there is a strong need for researchers to incorporate human postmortem brains in their study design. This approach could shed much needed light on precise neurobiological underpinnings of PTSD, and would allow one to assess the extent to which blood-based miRNA findings are informative to the CNS. In this context, it is becoming clear that focusing on exosome-associated biomarkers might provide interesting insights into the brain. Exosomes carry proteins and miRNAs, among others, as cargo from the cytosol of neurons to the extracellular space where they can be transported to the peripheral circulation by crossing the blood-brain barrier. As such, they can be accessed through the bloodstream, isolated and enriched for neuronal origin using neural-specific membrane markers [103, 104]. Recent studies have shown that $A\beta_{42}$ levels in blood exosomes derived from neurons, were higher in subjects with mild cognitive impairment (MCI), MCI that progressed to dementia, and AD [105]. In another study, blood exosomal levels of $A\beta_{42}$ and tau phosphorylated at Thr^{181} and Ser^{396} predicted the development of AD ten years before clinical onset [106]. Therefore, assessing the content of CNS-derived blood-based exosomes could be extremely informative. Alternatively, analyzing CSF could be equally useful in mirroring the neural environment more closely. Although more invasive, the collection and analysis of CSF-associated miRNAs could provide additional and valuable insights into pathological processes occurring within the brain. Similarly, examining several body fluids jointly, including plasma, serum, PBMCs, sperm and/or CSF, could further deepen our understanding of miRNA localization and overlap. Finally, the use of longitudinal designs could yield valuable information regarding dynamic changes over time and how these changes potentially relate to differential susceptibility to traumatic stress (Figure 2).

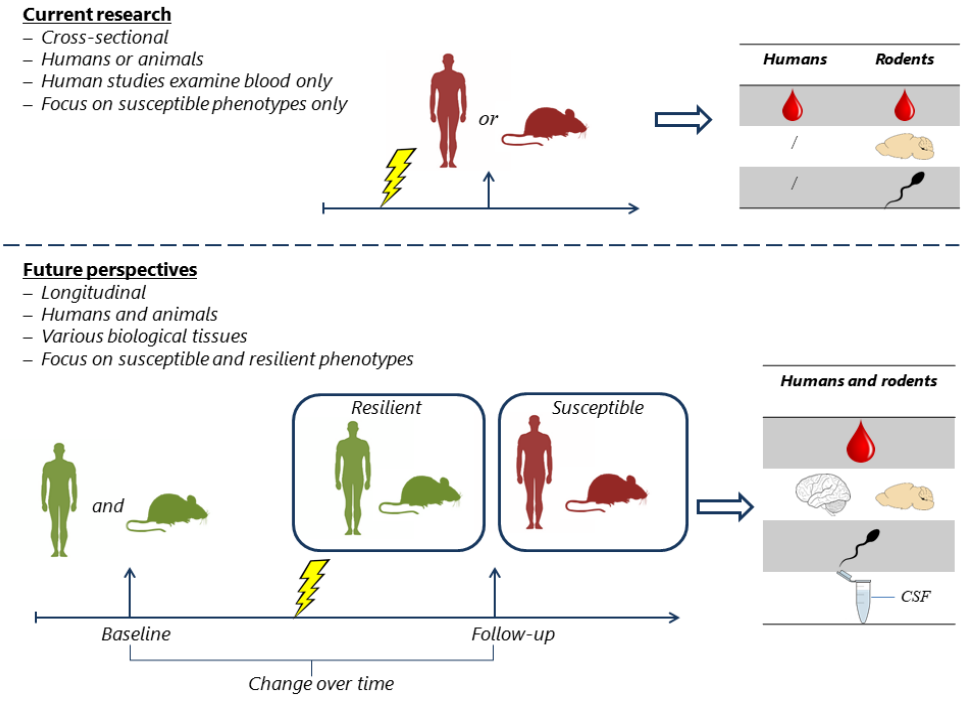

Figure 2. Current research and future perspectives for miRNA analyses in PTSD. Green and red silhouettes represent mental health or illness, respectively. The lightning bolt represents a traumatic event.

It is worth noting that guidelines such as the prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE) design [107] or the Strengthening the Reporting of Observational studies in Epidemiology for Molecular Epidemiology (STROBE-ME) [108] offer valuable overviews to help researchers in the design, execution and reporting of biomarker studies. With respect to analyzing miRNAs in particular, Nair *et al.* [109] recently provided a comprehensive overview of helpful study requirements for researchers involved in studying miRNAs in human diseases. Importantly, both human and animal studies have shown that differences in genetic backgrounds between subjects can have a considerable effect on the resolution of biomarker studies [110, 111]. Therefore, it is critical for future research to take variations in genetic backgrounds into account and correct for additional factors such as current or previous smoking habits, alcohol or medication use of patients.

Taken together, current preclinical and preliminary clinical evidence show some potential for the use of miRNAs as biomarkers for PTSD, which would enable us to detect at-risk individuals at an early stage and provide appropriate treatment options. This approach is especially relevant since to date, treatment options for PTSD are limited. Therefore, the presented findings lay a foundation for future research to further examine the exact roles of miRNAs in PTSD using appropriate study designs.

References

1. Schmidt, U., F. Holsboer, and T. Rein, *Epigenetic aspects of posttraumatic stress disorder*. Dis Markers, 2011. **30**(2-3): p. 77-87.
2. Association, A.P., *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
3. Smith, M.E., *Bilateral hippocampal volume reduction in adults with post-traumatic stress disorder: a meta-analysis of structural MRI studies*. Hippocampus, 2005. **15**(6): p. 798-807.
4. Karl, A., et al., *A meta-analysis of structural brain abnormalities in PTSD*. Neuroscience & Biobehavioral Reviews, 2006. **30**(7): p. 1004-1031.
5. Shin, L.M., S.L. Rauch, and R.K. Pitman, *Amygdala, medial prefrontal cortex, and hippocampal function in PTSD*. Ann N Y Acad Sci, 2006. **1071**: p. 67-79.
6. Etkin, A. and T.D. Wager, *Functional Neuroimaging of Anxiety: A Meta-Analysis of Emotional Processing in PTSD, Social Anxiety Disorder, and Specific Phobia*. The American Journal of Psychiatry, 2007. **164**(10): p. 1476-1488.
7. El Khoury-Malhame, M., et al., *Amygdala activity correlates with attentional bias in PTSD*. Neuropsychologia, 2011. **49**(7): p. 1969-1973.
8. Chrousos, G.P. and P.W. Gold, *The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis*. Jama, 1992. **267**(9): p. 1244-52.
9. Sherin, J.E. and C.B. Nemeroff, *Post-traumatic stress disorder: the neurobiological impact of psychological trauma*. Dialogues in Clinical Neuroscience, 2011. **13**(3): p. 263-278.
10. Sherin, J.E.N., Charles B., *Post-traumatic stress disorder: the neurobiological impact of psychological trauma*. Dialogues in Clinical Neuroscience, 2011. **13**(3).
11. Baker, D.G., et al., *Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder*. Am J Psychiatry, 1999. **156**(4): p. 585-8.
12. Yehuda, R., *Advances in understanding neuroendocrine alterations in PTSD and their therapeutic implications*. Ann N Y Acad Sci, 2006. **1071**: p. 137-66.
13. Geracioti, T.D., Jr., et al., *Effects of trauma-related audiovisual stimulation on cerebrospinal fluid norepinephrine and corticotropin-releasing hormone concentrations in post-traumatic stress disorder*. Psychoneuroendocrinology, 2008. **33**(4): p. 416-24.
14. Yehuda, R., *Biology of posttraumatic stress disorder*. J Clin Psychiatry, 2001. **62 Suppl 17**: p. 41-6.
15. Yehuda, R., et al., *Low cortisol and risk for PTSD in adult offspring of holocaust survivors*. Am J Psychiatry, 2000. **157**(8): p. 1252-9.
16. Meewisse, M.L., et al., *Cortisol and post-traumatic stress disorder in adults: systematic review and meta-analysis*. Br J Psychiatry, 2007. **191**: p. 387-92.
17. Young EA., B.N., *Cortisol and Catecholamines in Posttraumatic Stress Disorder: An Epidemiologic Community Study*. Arch Gen Psychiatry, 2004. **61**(4): p. 394-401.
18. Lemieux, A.M. and C.L. Coe, *Abuse-related posttraumatic stress disorder: evidence for chronic neuroendocrine activation in women*. Psychosom Med, 1995. **57**(2): p. 105-15.

19. Peschansky, V.J. and C. Wahlestedt, *Non-coding RNAs as direct and indirect modulators of epigenetic regulation*. Epigenetics, 2014. **9**(1): p. 3-12.
20. Kouzarides, T., *Chromatin Modifications and Their Function*. Cell, 2007. **128**(4): p. 693-705.
21. Venkatesh, S. and J.L. Workman, *Histone exchange, chromatin structure and the regulation of transcription*. Nat Rev Mol Cell Biol, 2015. **16**(3): p. 178-189.
22. Zamore, P.D., *Ancient pathways programmed by small RNAs*. Science, 2002. **296**(5571): p. 1265-9.
23. Memczak, S., et al., *Circular RNAs are a large class of animal RNAs with regulatory potency*. Nature, 2013. **495**(7441): p. 333-338.
24. Aravin, A.A., G.J. Hannon, and J. Brennecke, *The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race*. Science, 2007. **318**(5851): p. 761-4.
25. Fabian, M.R. and N. Sonenberg, *The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC*. Nat Struct Mol Biol, 2012. **19**(6): p. 586-93.
26. Pritchard, C.C., H.H. Cheng, and M. Tewari, *MicroRNA profiling: approaches and considerations*. Nat Rev Genet, 2012. **13**(5): p. 358-69.
27. Borchert, G.M., W. Lanier, and B.L. Davidson, *RNA polymerase III transcribes human microRNAs*. Nat Struct Mol Biol, 2006. **13**(12): p. 1097-1101.
28. Ramalingam, P., et al., *Biogenesis of intronic miRNAs located in clusters by independent transcription and alternative splicing*. RNA, 2014. **20**(1): p. 76-87.
29. Issler, O. and A. Chen, *Determining the role of microRNAs in psychiatric disorders*. Nat Rev Neurosci, 2015. **16**(4): p. 201-12.
30. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing*. Nature, 2003. **425**(6956): p. 415-9.
31. Davis-Dusenbery, B.N. and A. Hata, *Mechanisms of control of microRNA biogenesis*. J Biochem, 2010. **148**(4): p. 381-92.
32. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs*. Genome Res, 2009. **19**(1): p. 92-105.
33. Lewis, B.P., C.B. Burge, and D.P. Bartel, *Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets*. Cell, 2005. **120**(1): p. 15-20.
34. Yang, X., et al., *Both mature miR-17-5p and passenger strand miR-17-3p target TIMP3 and induce prostate tumor growth and invasion*. Nucleic Acids Research, 2013. **41**(21): p. 9688-9704.
35. Park, N.J., et al., *Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2009. **15**(17): p. 5473-5477.
36. Taylor, D. and C. Gercel-Taylor, *The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids*. Frontiers in Genetics, 2013. **4**(142).
37. Hanke, M., et al., *A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer*. Urol Oncol, 2010. **28**(6): p. 655-61.
38. Weber, J.A., et al., *The microRNA spectrum in 12 body fluids*. Clin Chem, 2010. **56**(11): p. 1733-41.
39. Turchinovich, A., et al., *Circulating miRNAs: cell-cell communication*

function? Frontiers in Genetics, 2013. **4**(119).

40. Camussi, G., et al., *Exosome/microvesicle-mediated epigenetic reprogramming of cells.* American Journal of Cancer Research, 2011. **1**(1): p. 98-110.
41. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nat Cell Biol, 2007. **9**(6): p. 654-659.
42. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
43. Vickers, K.C., et al., *MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins.* Nat Cell Biol, 2011. **13**(4): p. 423-33.
44. Wagner, J., et al., *Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs.* Arterioscler Thromb Vasc Biol, 2013. **33**(6): p. 1392-400.
45. Gibbings, D.J., et al., *Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity.* Nat Cell Biol, 2009. **11**(9): p. 1143-9.
46. Wang, K., et al., *Export of microRNAs and microRNA-protective protein by mammalian cells.* Nucleic Acids Research, 2010.
47. Etheridge, A., et al., *Extracellular microRNA: a new source of biomarkers.* Mutat Res, 2011. **717**(1-2): p. 85-90.
48. Smith, B., et al., *Large-scale expression analysis reveals distinct microRNA profiles at different stages of human neurodevelopment.* PLoS One, 2010. **5**(6): p. e11109.
49. Bian, S., T.-l. Xu, and T. Sun, *Tuning the cell fate of neurons and glia by microRNAs.* Current opinion in neurobiology, 2013. **23**(6): p. 10.1016/j.conb.2013.08.002.
50. Magill, S.T., et al., *microRNA-132 regulates dendritic growth and arborization of newborn neurons in the adult hippocampus.* Proc Natl Acad Sci U S A, 2010. **107**(47): p. 20382-7.
51. Dajas-Bailador, F., et al., *microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons.* Nat Neurosci, 2012.
52. Aksoy-Aksel, A., F. Zampa, and G. Schratt, *MicroRNAs and synaptic plasticity--a mutual relationship.* Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1652).
53. Hu, Z. and Z. Li, *miRNAs in synapse development and synaptic plasticity.* Current Opinion in Neurobiology, 2017. **45**: p. 24-31.
54. Smalheiser, N.R., et al., *MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects.* PLoS One, 2012. **7**(3): p. e33201.
55. Bai, M., et al., *Abnormal hippocampal BDNF and miR-16 expression is associated with depression-like behaviors induced by stress during early life.* PLoS One, 2012. **7**(10): p. e46921.
56. Absalon, S., et al., *MiR-26b, upregulated in Alzheimer's disease, activates cell cycle entry, tau-phosphorylation, and apoptosis in postmitotic neurons.* J Neurosci, 2013. **33**(37): p. 14645-59.
57. Hu, Y.K., et al., *MicroRNA-98 induces an Alzheimer's disease-like disturbance by targeting insulin-like growth factor 1.* Neurosci Bull, 2013. **29**(6): p. 745-51.
58. Wang, G., et al., *Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of alpha-synuclein.*

Am J Hum Genet, 2008. **82**(2): p. 283-9.
59. Doxakis, E., *Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153.* J Biol Chem, 2010. **285**(17): p. 12726-34.
60. Blechert, J., et al., *Fear conditioning in posttraumatic stress disorder: evidence for delayed extinction of autonomic, experiential, and behavioural responses.* Behav Res Ther, 2007. **45**(9): p. 2019-33.
61. Norberg, M.M., J.H. Krystal, and D.F. Tolin, *A meta-analysis of D-cycloserine and the facilitation of fear extinction and exposure therapy.* Biol Psychiatry, 2008. **63**(12): p. 1118-26.
62. Lin, Q., et al., *The brain-specific microRNA miR-128b regulates the formation of fear-extinction memory.* Nat Neurosci, 2011. **14**(9): p. 1115-7.
63. Konopka, W., et al., *MicroRNA loss enhances learning and memory in mice.* J Neurosci, 2010. **30**(44): p. 14835-42.
64. Dias, B.G., et al., *Amygdala-dependent fear memory consolidation via miR-34a and Notch signaling.* Neuron, 2014. **83**(4): p. 906-18.
65. Griggs, E.M., et al., *MicroRNA-182 Regulates Amygdala-Dependent Memory Formation.* The Journal of Neuroscience, 2013. **33**(4): p. 1734.
66. Vetere, G., et al., *Selective inhibition of miR-92 in hippocampal neurons alters contextual fear memory.* Hippocampus, 2014. **24**(12): p. 1458-65.
67. Jovasevic, V., et al., *GABAergic mechanisms regulated by miR-33 encode state-dependent fear.* Nat Neurosci, 2015. **18**(9): p. 1265-71.
68. Wang, R.Y., et al., *In vivo knockdown of hippocampal miR-132 expression impairs memory acquisition of trace fear conditioning.* Hippocampus, 2013. **23**(7): p. 625-33.
69. Bocchio-Chiavetto, L., et al., *Blood microRNA changes in depressed patients during antidepressant treatment.* Eur Neuropsychopharmacol, 2013. **23**(7): p. 602-11.
70. Lai, C.Y., et al., *MicroRNA expression aberration as potential peripheral blood biomarkers for schizophrenia.* PLoS One, 2011. **6**(6): p. e21635.
71. Rong, H., et al., *MicroRNA-134 plasma levels before and after treatment for bipolar mania.* Journal of Psychiatric Research. **45**(1): p. 92-95.
72. Balakathiresan, N.S., et al., *Serum and amygdala microRNA signatures of posttraumatic stress: fear correlation and biomarker potential.* J Psychiatr Res, 2014. **57**: p. 65-73.
73. Volk, N., et al., *MicroRNA-19b associates with Ago2 in the amygdala following chronic stress and regulates the adrenergic receptor beta 1.* J Neurosci, 2014. **34**(45): p. 15070-82.
74. Chen, R.J., et al., *MicroRNAs as biomarkers of resilience or vulnerability to stress.* Neuroscience, 2015. **305**: p. 36-48.
75. Rassoulzadegan, M., et al., *RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse.* Nature, 2006. **441**(7092): p. 469-74.
76. Liu, W.-M., et al., *Sperm-borne microRNA-34c is required for the first cleavage division in mouse.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(2): p. 490-494.
77. Gapp, K., et al., *Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice.* Nat Neurosci, 2014. **17**(5): p. 667-9.
78. Rodgers, A.B., et al., *Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation.* The

Journal of neuroscience : the official journal of the Society for Neuroscience, 2013. **33**(21): p. 9003-9012.
79. Binder, E.B., et al., *Association of FKBP5 polymorphisms and childhood abuse with risk of posttraumatic stress disorder symptoms in adults.* Jama, 2008. **299**(11): p. 1291-305.
80. Binder, E.B., et al., *Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment.* Nat Genet, 2004. **36**(12): p. 1319-25.
81. Mehta, D., et al., *Using polymorphisms in FKBP5 to define biologically distinct subtypes of posttraumatic stress disorder: evidence from endocrine and gene expression studies.* Arch Gen Psychiatry, 2011. **68**(9): p. 901-10.
82. Zannas, A.S., N. Provencal, and E.B. Binder, *Epigenetics of Posttraumatic Stress Disorder: Current Evidence, Challenges, and Future Directions.* Biol Psychiatry, 2015. **78**(5): p. 327-35.
83. Zimmermann, P., et al., *Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study.* Am J Psychiatry, 2011. **168**(10): p. 1107-16.
84. Klengel, T., et al., *Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions.* Nat Neurosci, 2013. **16**(1): p. 33-41.
85. Yehuda, R., et al., *Holocaust Exposure Induced Intergenerational Effects on FKBP5 Methylation.* Biol Psychiatry, 2016. **80**(5): p. 372-80.
86. Sabbagh, J.J., et al., *Age-Associated Epigenetic Upregulation of the FKBP5 Gene Selectively Impairs Stress Resiliency.* PLoS ONE, 2014. **9**(9): p. e107241.
87. Schmidt, M.V., et al., *The Prospect of FKBP51 as a Drug Target.* ChemMedChem, 2012. **7**(8): p. 1351-1359.
88. Zheng, D., et al., *MicroRNA-511 Binds to FKBP5 mRNA, Which Encodes a Chaperone Protein, and Regulates Neuronal Differentiation.* J Biol Chem, 2016. **291**(34): p. 17897-906.
89. Volk, N., et al., *Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress.* Cell Rep, 2016. **17**(7): p. 1882-1891.
90. Zhao, H., et al., *Neuroimmune modulation following traumatic stress in rats: evidence for an immunoregulatory cascade mediated by c-Src, miRNA222 and PAK1.* Journal of Neuroinflammation, 2011. **8**(1): p. 159.
91. Li, C., et al., *Dynamic Alterations of miR-34c Expression in the Hypothalamus of Male Rats after Early Adolescent Traumatic Stress.* Neural Plast, 2016. **2016**: p. 5249893.
92. Schmidt, U., et al., *Therapeutic Action of Fluoxetine is Associated with a Reduction in Prefrontal Cortical miR-1971 Expression Levels in a Mouse Model of Posttraumatic Stress Disorder.* Front Psychiatry, 2013. **4**: p. 66.
93. Cho, J.-H., et al., *Molecular evidence of stress-induced acute heart injury in a mouse model simulating posttraumatic stress disorder.* Proceedings of the National Academy of Sciences, 2014. **111**(8): p. 3188-3193.
94. Neigh, G.N. and F.F. Ali, *Co-morbidity of PTSD and immune system dysfunction: opportunities for treatment.* Current Opinion in Pharmacology, 2016. **29**: p. 104-110.
95. Michopoulos, V., et al., *Inflammation in Fear- and Anxiety-Based Disorders: PTSD, GAD, and Beyond.*

Neuropsychopharmacology, 2017. **42**(1): p. 254-270.
96. Gill, J.M., et al., *PTSD is associated with an excess of inflammatory immune activities.* Perspect Psychiatr Care, 2009. **45**(4): p. 262-77.
97. Daskalakis, N.P., et al., *New translational perspectives for blood-based biomarkers of PTSD: From glucocorticoid to immune mediators of stress susceptibility.* Exp Neurol, 2016. **284**(Pt B): p. 133-140.
98. Zhou, J., et al., *Dysregulation in microRNA expression is associated with alterations in immune functions in combat veterans with post-traumatic stress disorder.* PLoS One, 2014. **9**(4): p. e94075.
99. Bam, M., et al., *Evidence for Epigenetic Regulation of Pro-Inflammatory Cytokines, Interleukin-12 and Interferon Gamma, in Peripheral Blood Mononuclear Cells from PTSD Patients.* J Neuroimmune Pharmacol, 2016. **11**(1): p. 168-81.
100. Bam, M., et al., *Dysregulated immune system networks in war veterans with PTSD is an outcome of altered miRNA expression and DNA methylation.* Scientific Reports, 2016. **6**: p. 31209.
101. Martin, C.G., et al., *Circulating miRNA associated with posttraumatic stress disorder in a cohort of military combat veterans.* Psychiatry Res, 2017. **251**: p. 261-265.
102. Wingo, A.P., et al., *DICER1 and microRNA regulation in post-traumatic stress disorder with comorbid depression.* Nat Commun, 2015. **6**: p. 10106.
103. Kapogiannis, D., et al., *Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical AlzheimerБ─≥s disease.* The FASEB Journal, 2015. **29**(2): p. 589-596.
104. Goetzl, E.J., et al., *Altered lysosomal proteins in neural-derived plasma exosomes in preclinical Alzheimer disease.* Neurology, 2015. **85**(1): p. 40-7.
105. Winston, C.N., et al., *Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome protein profile.* Alzheimers Dement (Amst), 2016. **3**: p. 63-72.
106. Fiandaca, M.S., et al., *Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study.* Alzheimers Dement, 2015. **11**(6): p. 600-7.e1.
107. Pepe, M.S., et al., *Pivotal Evaluation of the Accuracy of a Biomarker Used for Classification or Prediction: Standards for Study Design.* JNCI Journal of the National Cancer Institute, 2008. **100**(20): p. 1432-1438.
108. Gallo, V., et al., *STrengthening the Reporting of OBservational studies in Epidemiology Б─⌠ Molecular Epidemiology STROBE-ME: an extension of the STROBE statement.* Journal of Clinical Epidemiology, 2011. **64**(12): p. 1350-1363.
109. Nair, V.S., et al., *Design and Analysis for Studying microRNAs in Human Disease: A Primer on -Omic Technologies.* Am J Epidemiol, 2014. **180**(2): p. 140-52.
110. Ahanda, M.-L.E., et al., *Impact of the Genetic Background on the Composition of the Chicken Plasma MiRNome in Response to a Stress.* PLOS ONE, 2014. **9**(12): p. e114598.
111. Zhao, H., et al., *A Pilot Study of Circulating miRNAs as Potential Biomarkers of Early Stage Breast Cancer.* PLOS ONE, 2010. **5**(10): p. e13735.

CHAPTER 4

Circulating serum microRNAs as potential diagnostic biomarkers of post-traumatic stress disorder: a pilot study

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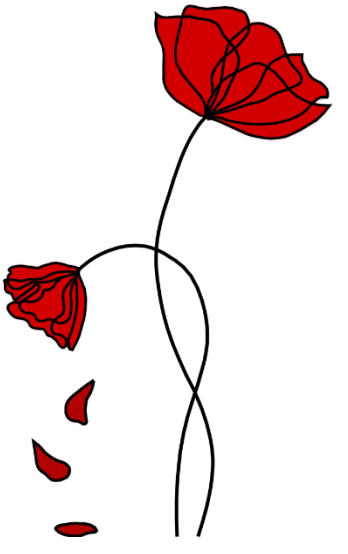


Abstract

Post-traumatic stress disorder (PTSD) is a psychiatric disorder that can develop upon exposure to a traumatic event. While most people are able to recover promptly, others are at increased risk of developing PTSD. However, the exact underlying biological mechanisms of differential susceptibility are unknown. Identifying biomarkers of PTSD could assist in its diagnosis and facilitate treatment planning. Here, we identified serum miRNAs of individuals who underwent a combat-related traumatic event and assessed their potential to serve as diagnostic biomarkers of PTSD. Next generation sequencing was performed to examine circulating miRNA profiles of 24 members belonging to the Dutch military cohort PRISMO. Three groups were selected: (i) susceptible subjects who developed PTSD after combat exposure, (ii) resilient subjects without PTSD, and (iii) non-exposed control subjects (N=8 per group). Differential expression analysis revealed 22 differentially expressed miRNAs in PTSD subjects compared to controls, and 1 in PTSD subjects compared to resilient individuals (after multiple testing correction and a log2 FC > |1|). Weighted gene co-expression network analysis (WGCNA) identified a module of co-expressed miRNAs, which could distinguish between the three groups. Receiver operating characteristic (ROC) curve analyses suggest that the miRNAs with the highest module membership could have a strong diagnostic accuracy as reflected by high areas under the curves. Overall, the results of our pilot study suggest that serum miRNAs could potentially serve as markers of PTSD, both individually or grouped within a cluster of co-expressed miRNAs. Larger studies are now needed to validate and build upon these preliminary findings.

Keywords: post-traumatic stress disorder, circulating microRNAs, diagnostic biomarker, trauma, susceptibility

Introduction

Post-traumatic stress disorder (PTSD) is a psychiatric disorder that can develop upon exposure to a life-threatening traumatic event, i.e. an event capable of producing intense feelings of fear, helplessness and horror [1]. Symptoms associated with PTSD include re-experiencing of the traumatic event, avoidance behavior, overall negative mood and hyperarousal [1]. Although approximately 60% of individuals within Western Europe will one day be exposed to a traumatic event, only about 6% of these develop PTSD, while others show a positive psychological adaptation process denoted as resilience [2-4]. However, some populations such as military soldiers are at elevated risk for trauma exposure, making PTSD a relatively common chronic disorder within the combat Veteran population [5]. There currently are limited treatment options for PTSD, without one being clearly superior to another [6]. Moreover, pharmacological treatment options for PTSD are at best moderately effective and only work for a subset of patients [7]. Therefore, increasing efforts are being made to unravel the biological underpinnings of PTSD in order to develop more efficient therapeutic strategies. It is now becoming clear that epigenetic mechanisms are involved in the lasting behavioral and molecular effects of trauma exposure [8, 9].

Epigenetics refers to a variety of processes that are triggered by environmental factors and cause lasting but reversible alterations in gene expression [10]. The non-coding microRNAs (miRNAs) are involved in post-transcriptional regulation of gene expression by binding to specific messenger RNAs (mRNAs) [11]. Several miRNAs have been found implicated in PTSD, shedding much needed light on the underlying pathophysiological underpinnings of this disorder [12-16]. Such findings emphasize the suggestion that expression profiles of miRNAs could potentially serve as relatively easily accessible biomarkers, or be embedded within a network of several relevant biological processes that, together, could more accurately reflect the complexity of PTSD. For those individuals who have difficulties recognizing or properly describing their symptoms, identifying such markers could be of use in clinical contexts in order to objectively confirm the presence of the disorder and establish appropriate treatment plans when needed [17]. Using these markers could be equally relevant during post-deployment medical screenings since military service members may have secondary reasons to not fully disclose their symptoms [18].

Here, we aimed to identify serum miRNAs associated with PTSD. We further aimed to gain insights in the co-expression patterns of these miRNAs, their predicted gene targets

and underlying biological pathways, along with their diagnostic accuracy. We hypothesized that specific miRNAs are differentially expressed between subjects with PTSD, trauma-exposed healthy individuals (referred to as "resilient" subjects here) and non-exposed healthy controls. To test this, we performed next generation sequencing (NGS) of 24 serum samples of military members belonging to a Dutch military cohort, and we compared miRNA profiles between the three groups. Our findings suggest that specific miRNAs, including miR-138-5p, are dysregulated in the circulation of individuals with PTSD.

Materials and methods

Participants

A subset of male military personnel (N=24) was selected from the larger Prospective Research In Stress-related Military Operations (PRISMO) study, a prospective cohort of Dutch military members deployed to Afghanistan [19, 20]. Based on the level of combat exposure during deployment and the severity of post-deployment PTSD symptoms, three subgroups were identified; (i) susceptible individuals, i.e. trauma-exposed subjects with deployment-related PTSD symptoms at 6 months follow-up, (ii) resilient individuals, i.e. trauma-exposed soldiers with no PTSD diagnosis at follow-up, and (iii) controls, i.e. deployed, but non-exposed and mentally healthy military members. Blood samples were collected at the Utrecht University Medical Center six months post-deployment. Trauma exposure was assessed using a 19-item deployment experiences checklist [21]. The severity of PTSD symptoms was established using the 22-item Self-Rating Inventory for PTSD (SRIP) [22]. Information on smoking and alcohol was collected using self-report measures. This study was approved by the ethical committee of the University Medical Center Utrecht (01-333/0) and was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent

RNA isolation

Total RNA was isolated from 300 µl human serum using the *mir*Vana PARIS kit (ThermoFisher) according to the manufacturer's instructions. Briefly, the samples were incubated with an equal volume of denaturing solution, Acid-Phenol:Chloroform was added and the samples spun for 5 min at 10,000x g. The aqueous phase was recovered and passed through a filter, which was washed three times with the provided wash solutions. Final RNA was eluted in 100 µl nuclease free water. The concentrations and quality of the recovered RNA were measured using the Agilent Bioanalyzer 2100 (Agilent Technologies). All eluates were stored at -80°C until further use.

Small RNA library preparation and next generation sequencing

Libraries (N=24, 8 per group) were prepared with an input of 25 ng total RNA using the Illumina Small RNA TruSeq kit (Illumina). Briefly, 3′ and 5′ RNA adapters were annealed, the samples were reverse transcribed and amplified using 11 PCR cycles. All samples were processed in parallel and received a unique barcode. The cDNA constructs were gel purified and concentrated by ethanol precipitation. The quality control was performed using Agilent's 2100 Bioanalyzer 2100 with a High Sensitivity DNA Chip. The 24 samples were pooled and sequenced in duplicate using the Illumina HiSeq 2000 DNA sequence platform according to the manufacturer's protocol (GEO accession: GSE137624).

Small RNA sequencing data analysis

Quality control of the raw sequences was done using FastQC (v. 0.11.3) and reads were preprocessed and mapped to the latest release of miRBase (v. 21) [23] utilizing miRge with default settings [24]. In order to compensate for bias introduced by very low abundant sequences, only those miRNAs with an average of 50 counts (or more) across samples were considered for further analyses.

Differential expression analysis

Data normalization and differential expression analysis was conducted with the DESeq2 package in R (v. 3.5.2) [25] while correcting for age, alcohol use and smoking status. Resulting *p*-values were controlled by the False Discovery Rate (FDR) at 5% [26].

Weighted gene co-expression network construction and module detection

The identified miRNAs were used to construct co-expression networks using the WGCNA R package [27]. Normalized miRNA data was used as input. An adjacency matrix was generated by calculating Pearson's correlations between all miRNAs. Topological overlap between miRNAs was calculated using a power of 9. 200 rounds of bootstrapping were performed in order to construct a network that is robust to outliers. The cutreeDynamic function in the dynamicTreeCut R package was used to identify coexpression modules of positively correlated miRNAs with high topological overlap. Modules with at least 30 miRNAs were assigned a color. Modules with highly correlated eigengenes were merged using the mergeCloseModules function in R. Pearson correlations between module eigengenes (ME), age, smoking status and alcohol were calculated. Welch's t-tests were performed in order to detect differences between MEs of the control subjects and the trauma-exposed individuals. One-way ANOVAs were performed to detect differences between the three groups. When significant, the post-hoc Tukey HSD test was used to detect pairwise group differences.

Target gene pathway and enrichment analyses

The experimentally validated miRNA-target interactions database miRTarBase 6.0 [28] was used to identify gene targets of miRNAs. In order to narrow down the amount of target genes used for further analyses, one-sided Fisher tests (with FDR multiple correction) were performed to evaluate whether the amount of miRNAs targeting a specific gene was significantly higher than expected by chance. Those genes were then analyzed for enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology terms (GO terms) using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 [29, 30].

Statistical analyses

To detect differences in age, number of previous deployments, cigarette smoking, alcohol use, trauma exposure scores and SRIP scores between the groups, Welch ANOVA with Games-Howell post-hoc tests were performed. Since data on alcohol use at the 6 months follow-up time point was not available for all subjects, pre-deployment values were used instead. For each individual, smoking status was estimated based on their methylation patterns in 183 CpGs, as previously described [31]. Finally, the classification accuracy of specific miRNAs was determined by calculating the area under the Receiver Operating Characteristic (ROC) curve (AUC) in R.

Results

Demographic characteristics

A total of 24 subjects were included in the present study of which 8 developed PTSD following deployment, 8 were resilient and 8 were non-exposed controls (Supplementary Table S1). Based on the sequencing results, 4 subjects were excluded due to having a distinctively lower amount of reads causing great variation in expression data between samples. The remaining three groups did not differ in terms of age, number of previous deployments, smoking status and alcohol use (Table 1). On average, subjects with PTSD and resilient individuals were exposed to a similar amount of traumatic events, which was significantly higher than the non-exposed controls ($F(2, 8.8) = 54.67$, $p<.001$. Games-Howell post-hoc showed $p<.001$ for PTSD vs control, and resilient vs control). Resilient and control subjects had similar post-deployment PTSD scores as measured by the SRIP which were significantly lower than the average score of the PTSD group ($F(2, 11.15)= 25.23$, $p<.001$. Games-Howell post-hoc showed $p<.001$ for PTSD vs resilient, and PTSD vs control).

Table 1. Demographic characteristics of the included subjects.

	Susceptible (N=8)	**Resilient (N=6)**	**Control (N=6)**	***P*-value**
Age when deployed	22.13 (0.61)	34.17 (4.88)	27.50 (3.62)	.083
Number of previous deployments	0.29 (0.18)	0.83 (0.48)	0.17 (0.17)	.465
Cigarette smoking	2.79 (1.65)	-1.43 (1.30)	-0.15 (1.89)	.194
Alcohol use	2.86 (0.67)	1.17 (0.40)	1.83 (0.60)	.155
Trauma exposure-score	7.75 (0.98)	7.17 (0.75)	0.5 (0.22)	<.001
SRIP PTSD score	55.25 (4.01)	25.50 (1.63)	24.50 (1.46)	<.001

Data is presented as mean (SE). SRIP: Self-Rating Inventory for Post-Traumatic Stress Disorder.

MicroRNA sequencing and differential expression analysis

Small RNA sequencing yielded an average of 9.5 million unfiltered sequencing reads across all samples. After adaptor trimming and size selection, an average of 1.9 million high-quality reads remained which were aligned to miRNA sequences from miRBase (release 21). The count data was filtered for those miRNAs showing an average of 50 reads or more across all samples. This resulted in 306 different miRNAs. Differential expression analysis in DESeq2 revealed that 123 miRNAs showed differential expression between PTSD cases and non-exposed controls, while 4 were downregulated in PTSD cases compared to resilient individuals (Supplementary Table S2 and S3). Selecting those miRNAs with a log2 fold-change (FC) value > |1.0| and FDR adjusted *p*-value <.05, revealed one miRNA, miR-1246, downregulated in PTSD subjects compared to resilient subjects and 22 differentially expressed miRNAs between PTSD subjects and non-exposed controls (Table 2, Figure 1). Of these, 4 were downregulated and 18 were upregulated.

Table 2. Differentially expressed miRNAs in PTSD cases vs controls and PTSD cases vs resilient individuals with a log2 fold-change value > |1.0| and FDR adjusted p-value <0.05.

PTSD vs control				**PTSD vs resilient**			
miRNA	**Log2 FC**	**P-value**	**FDR adj *P*-value**	**miRNA**	**Log2 FC**	**P-value**	**FDR adj *P*-value**
miR-218-2-3p	3.22	1.65E-02	4.20E-02	miR-1246	-1.06	3.54E-04	3.05E-02
miR-3609	3.05	8.22E-06	6.62E-05				
miR-432-5p	2.37	8.44E-04	3.31E-03				
miR-138-5p	2.29	2.18E-16	9.54E-15				
miR-221-5p	2.06	6.11E-13	1.34E-11				
miR-4485-3p	1.98	3.45E-15	9.59E-14				
miR-31-5p	1.92	2.16E-15	7.35E-14				
miR-146b-5p	1.67	1.86E-25	2.85E-23				
miR-5096	1.62	1.81E-06	1.84E-05				
miR-222-3p	1.56	2.81E-15	8.61E-14				
miR-1273g-3p	1.55	7.23E-05	3.75E-04				
miR-302a-5p	1.49	6.61E-09	1.06E-07				
miR-221-3p	1.45	1.03E-14	2.62E-13				
miR-619-5p	1.40	1.74E-04	8.31E-04				
miR-335-5p	1.28	7.18E-28	2.20E-25				
miR-146b-3p	1.25	6.30E-11	1.13E-09				
miR-3175	1.17	1.95E-04	9.20E-04				
miR-3656	1.00	1.07E-02	2.86E-02				
miR-184	-1.20	1.58E-05	1.01E-04				
let-7d-5p	-1.27	1.22E-18	9.33E-17				
miR-98-5p	-1.33	1.57E-22	1.60E-20				
miR-146a-5p	-2.04	1.52E-08	2.22E-07				

The table is organized based on decreasing log2 fold-change values. miRNA: microRNA, log2 FC: log2 fold-change. The hsa-prefix was removed due to space constraints.

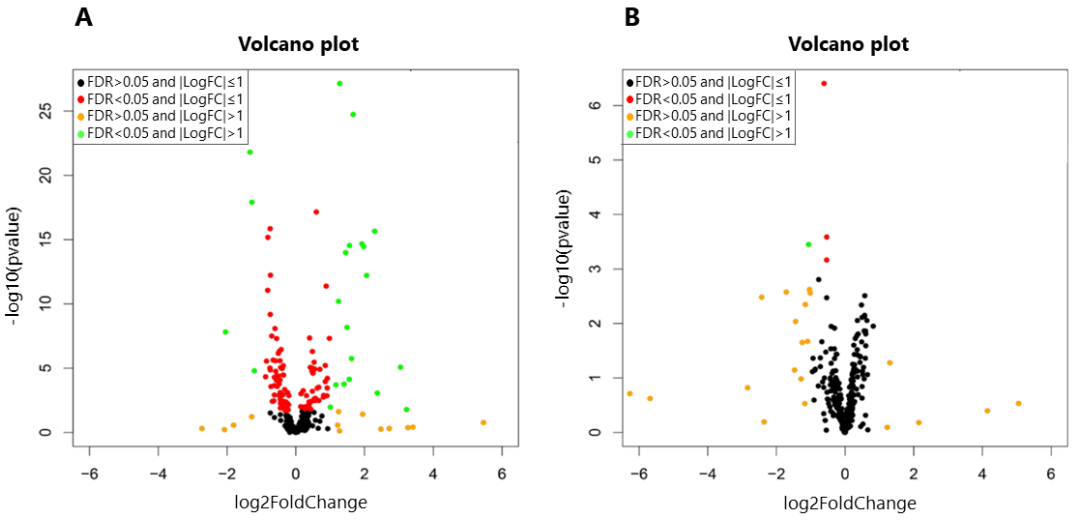


Figure 1. Volcano plots of differentially expressed miRNAs between PTSD cases and controls (A), and PTSD cases and resilient subjects (B). Black dots represent miRNAs with a log2 FC ≤ |1|, of which the expression is not significantly different between both groups. Orange dots represent miRNAs with a log2 FC > |1|, of which the expression is not significantly different between both groups. Red dots represent miRNAs with a log2 FC ≤ |1|, of which the expression is significantly different between both groups. Green dots represent differentially expressed miRNAs with a log2 FC > |1|. Significance is declared when adjusted p-value <0.05.

Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was applied using the 306 identified miRNAs in order to detect clusters of co-expressed miRNAs associated with PTSD status. Based on the sample dendrogram, one outlier was removed from further analyses (Supplementary Figure S1). We identified 3 miRNA modules (Figure 2). The turquoise, blue and brown modules had 84, 79 and 65 miRNAs, respectively. None of the modules were associated with the potential covariates age, smoking status or alcohol use (Supplementary Figure S2). The module eigengenes were significantly different between trauma-exposed individuals and non-exposed controls for the turquoise and blue modules (p = 2.67 x 10^{-04}, p = 2.51 x 10^{-06} respectively) but not for the brown module (p = 0.196; Figure 2A). When stratifying trauma-exposed individuals into PTSD subjects and resilient individuals, the individual eigengenes of the blue module were significantly different between PTSD subjects and resilient individuals (p = 1.46 x 10^{-03}; Figure 2B) which was not the case for the other modules. We therefore focused on the miRNAs within blue module for further downstream analyses.

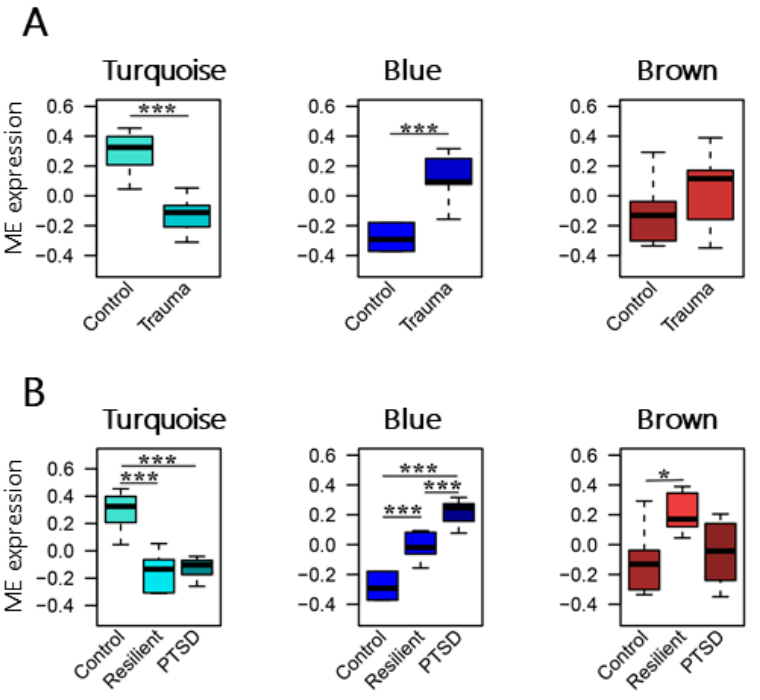


Figure 2. Modules of co-expressed miRNAs showing module eigengene expression levels (y-axis) across the groups (x-axis). Groups are stratified by trauma exposure (A), or trauma exposure and PTSD status (B). Significances were detected using Welch tests (A) or one-way ANOVAs (B). * indicates $p<0.05$, *** indicates $p<.001$. ME: module eigengenes.

Out of the 79 miRNAs belonging to this module (Table 3), 67 were differentially expressed between PTSD subjects and controls, including miR-138-5p, the hub miRNA of this module. In order to evaluate the diagnostic accuracy of some of these miRNAs, we performed ROC analysis for the miRNAs with the highest absolute module memberships. The 5 most contributing miRNAs, i.e. miR-221-3p, miR-335-5p, miR-138-5p, miR-222-3p and miR-146-5p (Table 3), could perfectly distinguish PTSD subjects and controls (AUC of 1 for all miRNAs; Supplementary Figure S3 A.1 and A.2 as an example for miR-221-3p). These miRNAs could equally well differentiate PTSD subjects from resilient subjects, except for miR-221-3p and miR-222-3p (AUCs of 0.95 and 0.98, respectively). When obtaining ROC curves using miRNA expression levels adjusted for confounders (i.e. age, smoking and alcohol use), all miRNAs could still distinguish PTSD subjects from controls (Supplementary Figure S3 B.1 and B.2 for miR-221-3p). However, differentiating PTSD from resilience was less accurate as reflected by AUCs of 0.625, 0.775, 0.725, 0.675, 0.775 for miR-221-3p, miR-335-5p, miR-138-5p, miR-222-3p and miR-146-5p, respectively (Supplementary Figure S3 B.1 and B.2 for miR-221-3p).

Table 3. MicroRNAs belonging to the blue module (WGCNA).

Nr	miRNA	Nr	miRNA	Nr	miRNA
1	hsa-miR-221-3p	28	hsa-miR-148a-3p	55	hsa-miR-20a-5p
2	hsa-miR-335-5p	29	hsa-miR-708-5p	56	hsa-miR-455-5p
3	hsa-miR-138-5p	30	hsa-miR-34a-5p	57	hsa-miR-874-5p
4	hsa-miR-222-3p	31	hsa-miR-574-3p	58	hsa-miR-675-5p
5	hsa-miR-146b-5p	32	hsa-miR-145-3p	59	hsa-miR-504-5p
6	hsa-miR-31-5p	33	hsa-miR-490-5p	60	hsa-miR-654-3p*
7	hsa-miR-340-5p	34	hsa-miR-148b-5p	61	hsa-miR-30a-3p*
8	hsa-miR-210-3p	35	hsa-miR-143-3p	62	hsa-miR-425-3p
9	hsa-miR-208b-3p	36	hsa-miR-184	63	hsa-miR-183-5p
10	hsa-miR-302a-5p	37	hsa-miR-199b-3p	64	hsa-miR-532-3p*
11	hsa-let-7i-5p	38	hsa-miR-628-5p	65	hsa-miR-193b-3p*
12	hsa-miR-4454	39	hsa-miR-132-3p	66	hsa-miR-130a-3p
13	hsa-miR-146b-3p	40	hsa-miR-181c-3p	67	hsa-miR-7151-5p*
14	hsa-miR-99b-3p	41	hsa-miR-641	68	hsa-miR-23a-3p
15	hsa-let-7a-3p	42	hsa-miR-208a-3p	69	hsa-miR-877-3p*
16	hsa-miR-221-5p	43	hsa-miR-18a-3p	70	hsa-miR-424-3p
17	hsa-miR-27a-3p/27b-3p	44	hsa-miR-193a-5p	71	hsa-miR-151a-3p
18	hsa-miR-127-3p	45	hsa-miR-411-5p*	72	hsa-miR-3175
19	hsa-miR-3200-3p	46	hsa-miR-148a-5p	73	hsa-miR-361-3p
20	hsa-miR-128-3p	47	hsa-miR-505-3p	74	hsa-miR-92b-5p
21	hsa-miR-20b-5p	48	hsa-miR-214-3p	75	hsa-miR-181a-3p
22	hsa-miR-199a-3p	49	hsa-miR-335-3p	76	hsa-miR-328-3p*
23	hsa-miR-181c-5p	50	hsa-miR-4485-3p	77	hsa-miR-423-3p*
24	hsa-miR-652-3p	51	hsa-miR-10a-5p	78	hsa-miR-139-5p*
25	hsa-miR-4662a-5p	52	hsa-miR-212-3p	79	hsa-miR-339-3p*
26	hsa-miR-17-5p/106a-5p	53	hsa-miR-331-3p		
27	hsa-miR-146a-5p	54	hsa-miR-490-3p*		

MicroRNAs with an asterisk were *not* differentially expressed in PTSD cases vs controls or PTSD vs resilient subjects. The microRNAs are ranked based on their absolute module membership values (highest to lowest).

Target gene pathway and GO enrichment analyses

Gene targets of the 79 miRNAs of the blue module were obtained from the online database miRTarBase [28]. In order to narrow down the extensive set of target genes (N=9270), Fisher tests were performed to select only those genes that were targeted by significantly more miRNAs than expected by chance. This yielded 146 genes, which were used for pathway and enrichment analyses (Supplementary Table S4). After FDR adjustment, 15 significantly enriched KEGG pathways were identified, of which most were cancer-related (Table 4). GO enrichment analyses of these target genes identified 8 significant biological processes (BP; Supplementary Table S5), 5 molecular functions (MF; Supplementary Table S6) and 6 cellular components (CC; Supplementary Table S7). The most enriched GO-terms were related to apoptotic processes, protein binding, and intracellular compartments, respectively.

Table 4. Significant KEGG Pathways enriched for a subset of target genes (N=146) of the co-expressed miRNAs from the blue module (N=79).

Term	***P*-value** ***Genes***	**FDR adj *p*-value**
Pathways in cancer	2.94E-10	3.66E-07
	RB1, SMAD2, EGLN3, SMAD4, SMAD3, MMP2, PTEN, PIK3R3, PTGS2, ETS1, EGFR, NFKB1, MYC, AKT2, KIT, E2F1, BCL2, GNAS, BAX, E2F2, AKT1, PPARG, MET, FGF11	
Pancreatic cancer	6.27E-09	7.79E-06
	RB1, SMAD2, SMAD4, SMAD3, AKT2, E2F1, PIK3R3, E2F2, AKT1, EGFR, NFKB1	
HTLV-I infection	1.26E-08	1.56E-05
	RB1, SMAD2, MAP3K3, SMAD4, SMAD3, TRRAP, PIK3R3, ETS1, TNF, NFKB1, PPP3R1, MYC, AKT2, E2F1, BAX, E2F2, AKT1, MAP3K14	
Small cell lung cancer	8.93E-08	1.11E-04
	RB1, AKT2, MYC, PTEN, BCL2, E2F1, PIK3R3, E2F2, AKT1, PTGS2, NFKB1	
Melanoma	2.14E-07	2.65E-04
	RB1, AKT2, PTEN, E2F1, PIK3R3, E2F2, AKT1, MET, FGF11, EGFR	
Hepatitis B	2.23E-07	2.77E-04
	RB1, SMAD4, PTEN, PIK3R3, TNF, NFKB1, MYC, AKT2, E2F1, BCL2, BAX, E2F2, AKT1	
FoxO signaling pathway	8.06E-07	1.00E-03
	SMAD2, SMAD4, BCL2L11, SMAD3, AKT2, BNIP3, PTEN, IRS4, PIK3R3, AKT1, SIRT1, EGFR	
Colorectal cancer	9.16E-07	1.14E-03
	SMAD2, SMAD4, SMAD3, AKT2, MYC, BCL2, BAX, PIK3R3, AKT1	
Prostate cancer	1.38E-06	1.71E-03
	RB1, AKT2, PTEN, BCL2, E2F1, PIK3R3, E2F2, AKT1, EGFR, NFKB1	
MicroRNAs in cancer	2.34E-06	2.91E-03
	BMPR2, PTEN, BRCA1, PTGS2, SIRT1, EGFR, NFKB1, ZEB2, BCL2L11, MYC, STMN1, E2F1, BCL2, E2F2, MET, BCL2L2	
Chronic myeloid leukemia	2.93E-06	3.63E-03
	RB1, SMAD4, AKT2, MYC, E2F1, PIK3R3, E2F2, AKT1, NFKB1	
Apoptosis	1.14E-05	1.41E-02
	AKT2, BCL2, BAX, PIK3R3, AKT1, TNF, MAP3K14, NFKB1	
Central carbon metabolism in cancer	1.41E-05	1.75E-02
	AKT2, MYC, KIT, PTEN, PIK3R3, AKT1, MET, EGFR	
MAPK signaling pathway	1.49E-05	1.85E-02
	MAP3K3, TNF, EGFR, NFKB1, PPP3R1, MYC, RASA1, AKT2, STMN1, FLNA, AKT1, MAP3K14, HSPA1B, FGF11	
Glioma	1.56E-05	1.94E-02
	RB1, AKT2, PTEN, E2F1, PIK3R3, E2F2, AKT1, EGFR	

Pathways were identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Discussion

In this study, we aimed to identify circulating miRNA profiles associated with PTSD using serum samples from Dutch military subjects. We further aimed to gain insights in the co-expression patterns of these miRNAs, their predicted gene targets and underlying biological pathways. Our preliminary findings suggest that (i) specific miRNAs are dysregulated between subjects with PTSD, resilient individuals and healthy controls, and (ii) the co-expression of a specific set of miRNAs could accurately distinguish between subjects with PTSD, resilient individuals and non-exposed controls. Once validated, such miRNAs could potentially be useful in clinical settings for accurate diagnosis, which is especially relevant for individuals who have difficulties associating their symptoms to a traumatic event, are unable to describe their symptoms or are unwilling to fully disclose them [18].

Differential expression analysis identified one differentially expressed miRNA between subjects with PTSD and resilient individuals and 22 between subjects with PTSD and non-exposed controls (after multiple testing correction and a log2 FC cutoff > |1|). Of these 22, miR-138-5p was significantly overexpressed in subjects with PTSD as compared to controls, and WGCNA revealed that this miRNA was the hub miRNA of the most relevant module. Among other functions, miR-138-5p has been found implicated in the regulation of dendritic spine morphology in mouse hippocampal neurons [32], and the proliferation and differentiation of neural stem cells [33]. Previous studies found altered serum levels of miR-138-5p in a rat model of restraint stress [34], and associations of hippocampal miR-138-5p levels with the formation of fear memories in mice [35]. Human studies further confirmed its implication in memory functions [36]. Another miRNA, miR-1246, was the only miRNA that was significantly downregulated in PTSD cases compared to resilient subjects and had a log2 FC larger than |1|. Although little is known regarding its function, this miRNA was previously also found downregulated in peripheral blood mononuclear cells (PBMCs) of war veterans suffering from PTSD as compared to healthy non trauma-exposed controls [14]. Our findings now provide further evidence for the implication of these two miRNAs in PTSD-related phenotypes.

The three modules of co-expressed miRNAs identified by WGCNA revealed that most of the detected miRNAs could be clustered based on similarities in their expression profiles. The blue module contained 79 miRNAs which could significantly differentiate trauma-exposed individuals from non-exposed controls. Interestingly, within the trauma-exposed individuals, the expression profiles of these miRNAs were significantly different between

individuals with and without PTSD. This highlights the importance of including and studying not only non-trauma exposed controls, but also trauma-exposed healthy individuals in order to disentangle PTSD effects from trauma-related effects. Moreover, 67 of the miRNAs of the blue module, including its hub miRNA, were significantly differentially expressed between PTSD cases and controls, which enhances the notion that this cluster of miRNAs could be relevant for PTSD.

Of the 5 miRNAs with the highest module membership, we calculated the AUCs to assess their diagnostic accuracy [37]. In order to determine the biomarker potential of these miRNAs, i.e. their potential to reflect PTSD regardless of any other confounding condition such as smoking and alcohol use, we used uncorrected miRNA expression values. The results suggest that these miRNAs could almost perfectly distinguish PTSD subjects from resilient individuals and controls. However, these results were not reflected by the DESeq2 analyses in which the expression levels of these particular miRNAs were not different between PTSD cases and resilient individuals. Part of this discrepancy can most likely be attributed to confounding effects, as DESeq2 analyses were corrected for age, alcohol and smoking status. When obtaining the ROC curves using confounder-adjusted miRNA expression values, the AUCs more accurately reflected the DESeq2 results. Although these results suggest that expression profiles of our selected miRNAs fluctuate with confounders, they mostly strengthen the need for replication in larger cohorts since small sample sizes are known to be associated with less precise ROC estimations [38]. This will further be valuable in determining whether these miRNAs could be specific for PTSD only as opposed to trauma more broadly.

Enrichment of GO-terms indicated that the target genes of the co-expressed miRNAs in the blue module are enriched in several KEGG pathways of which most are cancer-related. This suggests that these miRNAs could be implicated in cancer pathways that are also involved in signaling cascades possibly related to PTSD. The target genes were also involved in several biological processes of which most were involved in apoptosis. Previous studies found reduced level of apoptotic markers in the serum of subjects with PTSD [39] and abnormal apoptosis in specific brain regions of animals undergoing single prolonged stress as a model for PTSD [40-42]. These findings indicate a potential apoptosis dysfunction that could contribute to the inflammation pattern frequently observed within PTSD [39]. Furthermore, the involvement of the identified genes in cellular responses after mechanical stimuli could indicate the need to correct for traumatic brain injuries (TBI) which are not uncommon among military members. Unfortunately, this information was not available for the present study. Finally, enriched

GO-terms for molecular function suggest their involvement in the binding of proteins and RNA, while the significant cellular component GO-terms point to intracellular compartments such as the cytosol and the nucleoplasm.

The present paper refers to trauma-exposed healthy individuals as being "resilient" in order to create a clear differentiation between trauma-exposed healthy subjects and non-exposed control subjects. However, we do acknowledge and emphasize that resilience is more than just the reverse side of PTSD or the absence of symptomatology [2, 43]. Instead, resilience is an active and dynamic process that needs to remain separated from the multifaceted and complex nature of PTSD. This complexity further suggests that identifying one true and valid biomarker of susceptibility is likely not realistic. We therefore urge future studies to combine findings such as the ones presented in this paper with several other biological networks and phenotypic profiles in order to obtain a more global and cross-dimensional understanding of PTSD.

The main strength of this study lies in the inclusion of three different groups, i.e. PTSD subjects, resilient subjects and non-exposed healthy controls, which allows one to disentangle PTSD- from trauma-related effects. However, the study is mainly limited by its small sample size consisting of male subjects only. Given the presence of female- and male-biased miRNAs, as recently reported by Cui, Yang et al. (2018) [44], these findings may not be generalizable to the female population. This study population may also differ from other cohorts such as civilians in terms of demographics, psychological characteristics, and types of experienced trauma, which further limits its extrapolation potential. Finally, one could question the validity of self-report PTSD measures and whether the observed markers are specific to PTSD since certain comorbidities such as (history of) TBI were not available and thus not accounted for.

In conclusion, this study presents preliminary evidence for the dysregulation of specific blood-based miRNAs in PTSD, including miR-138-5p, which is known to be involved in key processes within the central nervous system, and has previously been found associated with stress-related phenotypes. Building up on these findings in order to identify reliable biomarkers for PTSD will be essential for accurate diagnosis and treatment planning. We therefore encourage future studies to further validate these findings using larger cohorts and thus pave the way for functional studies to gain insights into the precise roles of these miRNAs in stress susceptibility.

References

1. Association, A.P., *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
2. Kalisch, R., et al., *The resilience framework as a strategy to combat stress-related disorders.* Nature Human Behaviour, 2017. **1**(11): p. 784-790.
3. Koenen, K.C., et al., *Posttraumatic stress disorder in the World Mental Health Surveys.* Psychol Med, 2017. **47**(13): p. 2260-2274.
4. Koenen, K.C., et al., *Posttraumatic stress disorder in the World Mental Health Surveys.* Psychological medicine, 2017. **47**(13): p. 2260-2274.
5. Thomas, M.M., et al., *Mental and Physical Health Conditions in US Combat Veterans: Results From the National Health and Resilience in Veterans Study.* Prim Care Companion CNS Disord, 2017. **19**(3).
6. Yehuda, R., et al., *PTSD in the military: special considerations for understanding prevalence, pathophysiology and treatment following deployment.* Eur J Psychotraumatol, 2014. **5**.
7. Richter-Levin, G., O. Stork, and M.V. Schmidt, *Animal models of PTSD: a challenge to be met.* Molecular Psychiatry, 2018.
8. Schmidt, U., F. Holsboer, and T. Rein, *Epigenetic aspects of posttraumatic stress disorder.* Dis Markers, 2011. **30**(2-3): p. 77-87.
9. Snijders, C., et al., *MicroRNAs in Post-traumatic Stress Disorder.* Curr Top Behav Neurosci, 2018. **38**: p. 23-46.
10. Goldberg, A.D., C.D. Allis, and E. Bernstein, *Epigenetics: a landscape takes shape.* Cell, 2007. **128**(4): p. 635-8.
11. Peschansky, V.J. and C. Wahlestedt, *Non-coding RNAs as direct and indirect modulators of epigenetic regulation.* Epigenetics, 2014. **9**(1): p. 3-12.
12. Bam, M., et al., *Evidence for Epigenetic Regulation of Pro-Inflammatory Cytokines, Interleukin-12 and Interferon Gamma, in Peripheral Blood Mononuclear Cells from PTSD Patients.* J Neuroimmune Pharmacol, 2016. **11**(1): p. 168-181.
13. Zhou, J., et al., *Dysregulation in microRNA expression is associated with alterations in immune functions in combat veterans with post-traumatic stress disorder.* PLoS One, 2014. **9**(4): p. e94075.
14. Bam, M., et al., *Dysregulated immune system networks in war veterans with PTSD is an outcome of altered miRNA expression and DNA methylation.* Sci Rep, 2016. **6**: p. 31209.
15. Martin, C.G., et al., *Circulating miRNA associated with posttraumatic stress disorder in a cohort of military combat veterans.* Psychiatry Res, 2017. **251**: p. 261-265.
16. Wingo, A.P., et al., *DICER1 and microRNA regulation in post-traumatic stress disorder with comorbid depression.* Nat Commun, 2015. **6**: p. 10106.
17. Lehrner, A. and R. Yehuda, *Biomarkers of PTSD: military applications and considerations.* Eur J Psychotraumatol, 2014. **5**.
18. Yehuda, R., et al., *The use of biomarkers in the military: from theory to practice.* Psychoneuroendocrinology, 2013. **38**(9): p. 1912-22.
19. Reijnen, A., et al., *Prevalence of mental health symptoms in Dutch military personnel returning from deployment to Afghanistan: a 2-year longitudinal analysis.* Eur Psychiatry, 2015. **30**(2): p. 341-6.

20. Eekhout, I., et al., *Post-traumatic stress symptoms 5 years after military deployment to Afghanistan: an observational cohort study.* Lancet Psychiatry, 2016. **3**(1): p. 58-64.
21. van Zuiden, M., et al., *Pre-existing high glucocorticoid receptor number predicting development of posttraumatic stress symptoms after military deployment.* Am J Psychiatry, 2011. **168**(1): p. 89-96.
22. Hovens, J.E., I. Bramsen, and H.M. van der Ploeg, *Self-rating inventory for posttraumatic stress disorder: review of the psychometric properties of a new brief Dutch screening instrument.* Percept Mot Skills, 2002. **94**(3 Pt 1): p. 996-1008.
23. Kozomara, A. and S. Griffiths-Jones, *miRBase: annotating high confidence microRNAs using deep sequencing data.* Nucleic Acids Res, 2014. **42**(Database issue): p. D68-73.
24. Baras, A.S., et al., *miRge - A Multiplexed Method of Processing Small RNA-Seq Data to Determine MicroRNA Entropy.* PLOS ONE, 2015. **10**(11): p. e0143066.
25. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
26. Benjamini, Y., Hochberg, Y., *Controlling The False Discovery Rate: A Practical And Powerful Approach To Multiple Testing.* Journal of the Royal Statistical Society, 1995. **57**: p. 289-300.
27. Langfelder, P. and S. Horvath, *WGCNA: an R package for weighted correlation network analysis.* BMC Bioinformatics, 2008. **9**: p. 559.
28. Chou, C.H., et al., *miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions.* Nucleic Acids Res, 2018. **46**(D1): p. D296-D302.
29. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.
30. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists.* Nucleic Acids Res, 2009. **37**(1): p. 1-13.
31. Zeilinger, S., et al., *Tobacco smoking leads to extensive genome-wide changes in DNA methylation.* PLoS One, 2013. **8**(5): p. e63812.
32. Siegel, G., et al., *A functional screen implicates microRNA-138-dependent regulation of the depalmitoylation enzyme APT1 in dendritic spine morphogenesis.* Nat Cell Biol, 2009. **11**(6): p. 705-16.
33. Wang, J., et al., *MicroRNA1385p regulates neural stem cell proliferation and differentiation in vitro by targeting TRIP6 expression.* Mol Med Rep, 2017. **16**(5): p. 7261-7266.
34. Balakathiresan, N.S., et al., *Serum and amygdala microRNA signatures of posttraumatic stress: Fear correlation and biomarker potential.* Journal of Psychiatric Research, 2014. **57**: p. 65-73.
35. Li, D.W., et al., *Fear conditioning downregulates miR-138 expression in the hippocampus to facilitate the formation of fear memory.* Neuroreport, 2018. **29**(16): p. 1418-1424.
36. Schroder, J., et al., *MicroRNA-138 is a potential regulator of memory performance in humans.* Front Hum Neurosci, 2014. **8**: p. 501.
37. Grund, B. and C. Sabin, *Analysis of biomarker data: logs, odds ratios, and receiver operating characteristic curves.* Current opinion in HIV and AIDS, 2010. **5**(6): p. 473-479.

38. Min, L., et al., *Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs*. J Extracell Vesicles, 2019. **8**(1): p. 1643670.
39. Mkrtchian, G.M., et al., *The involvement of abnormal apoptosis in the disturbance of synaptic plasticity in posttraumatic stress disorder*. Zh Nevrol Psikhiatr Im S S Korsakova, 2013. **113**(1): p. 26-9.
40. Jia, Y., et al., *Role of apoptosis in the Post-traumatic stress disorder model-single prolonged stressed rats*. Psychoneuroendocrinology, 2018. **95**: p. 97-105.
41. Han, F., S. Yan, and Y. Shi, *Single-prolonged stress induces endoplasmic reticulum-dependent apoptosis in the hippocampus in a rat model of post-traumatic stress disorder*. PLoS One, 2013. **8**(7): p. e69340.
42. Li, Y., F. Han, and Y. Shi, *Increased Neuronal Apoptosis in Medial Prefrontal Cortex is Accompanied with Changes of Bcl-2 and Bax in a Rat Model of Post-Traumatic Stress Disorder*. Journal of Molecular Neuroscience, 2013. **51**(1): p. 127-137.
43. Snijders, C., et al., *Resilience Against Traumatic Stress: Current Developments and Future Directions*. 2018. **9**(676).
44. Cui, C., et al., *Identification and Analysis of Human Sex-biased MicroRNAs*. Genomics Proteomics Bioinformatics, 2018. **16**(3): p. 200-211.

Supplementary material

Supplementary Table S1. Demographic characteristics of 24 subjects before outlier exclusion.

	Susceptible (N=8)	**Resilient (N=8)**	**Control (N=8)**	***P*-value**
Age when deployed	22.13 (0.61)	32.75 (4.03)	26.75 (2.71)	.047
Number of previous deployments	0.29 (0.18)	1.13 (0.55)	0.25 (0.16)	.358
Military rank	1.38 (0.26)	2.13 (0.40)	1.5 (0.27)	.222
Cigarette smoking at baseline	2 (0.62)	0.38 (0.26)	0.38 (0.38)	.093
Alcohol use	2.86 (0.67)	1.25 (0.31)	1.88 (0.44)	.130
Trauma exposure-score	7.75 (0.98)	7.75 (0.67)	0.5 (0.05)	<.001
SRIP PTSD score	55.25 (4.00)	25.38 (1.32)	24.50 (1.17)	<.001

Data is presented as mean (SE). SRIP: Self-Rating Inventory for Post-traumatic stress disorder.

Supplementary Table S2. Differentially expressed miRNAs in PTSD subjects vs controls with FDR adjusted *p*-values <0.05.

miRNA	***Log2 FC***	***FDR adj* P-*value***	***miRNA***	***Log2 FC***	***FDR adj* P-*value***
miR-218-2-3p	3.22	4.20E-02	miR-30c-1-3p	-0.22	4.20E-02
miR-3609	3.04	6.62E-05	miR-23b-3p	-0.24	3.18E-03
miR-432-5p	2.37	3.31E-03	miR-874-5p	-0.25	4.69E-02
miR-138-5p	2.29	9.54E-15	miR-26a-5p	-0.25	1.57E-02
miR-221-5p	2.06	1.34E-11	miR-454-5p	-0.27	2.34E-02
miR-4485-3p	1.98	9.59E-14	miR-675-3p	-0.29	1.25E-02
miR-31-5p	1.92	7.35E-14	miR-652-3p	-0.30	2.47E-02
miR-146b-5p	1.67	2.85E-23	miR-210-5p	-0.32	4.61E-02
miR-5096	1.62	1.84E-05	miR-361-3p	-0.33	1.96E-03
miR-222-3p	1.56	8.61E-14	miR-181c-3p	-0.33	1.94E-02
miR-1273g-3p	1.55	3.75E-04	miR-191-5p	-0.33	3.26E-02
miR-302a-5p	1.49	1.06E-07	miR-301b-3p	-0.36	1.97E-04
miR-221-3p	1.45	2.62E-13	miR-1287-5p	-0.36	2.69E-02
miR-619-5p	1.40	8.31E-04	miR-181a-2-3p	-0.36	5.16E-05
miR-335-5p	1.28	2.20E-25	miR-340-5p	-0.37	1.97E-04
miR-146b-3p	1.25	1.13E-09	miR-143-5p	-0.38	4.52E-03
miR-3175	1.17	9.20E-04	miR-378a-3p/378c/378d/378e	-0.39	1.10E-02
miR-3656	1.00	2.86E-02	miR-30d-5p	-0.40	7.38E-05
miR-20b-5p	0.98	5.93E-07	miR-92b-5p	-0.42	2.43E-02
miR-214-3p	0.92	3.27E-04	miR-27a-3p/27b-3p	-0.43	4.15E-06
miR-193a-5p	0.91	1.49E-03	miR-181b-5p	-0.44	6.62E-05
miR-335-3p	0.91	4.93E-03	miR-2110	-0.45	3.26E-02
miR-208b-3p	0.88	8.32E-11	miR-1180-3p	-0.45	1.57E-02
miR-34a-5p	0.86	5.16E-05	miR-342-3p	-0.45	8.76E-03

miR-18a-3p	0.86	5.37E-04	miR-125a-5p	-0.46	3.18E-03
miR-1246	0.81	4.79E-03	miR-628-5p	-0.46	3.18E-03
miR-127-3p	0.80	6.39E-03	miR-7706	-0.46	4.17E-04
miR-29a-3p	0.70	8.13E-05	miR-130b-5p	-0.46	2.46E-05
miR-324-3p	0.67	1.06E-02	let-7i-5p	-0.48	5.46E-06
miR-675-5p	0.67	1.33E-03	miR-148b-5p	-0.49	4.72E-03
miR-490-5p	0.62	1.52E-03	miR-143-3p	-0.49	8.01E-04
miR-641	0.60	1.04E-02	let-7a-5p/7c-5p	-0.51	7.24E-06
miR-532-5p	0.59	4.35E-16	miR-148a-3p	-0.51	2.21E-04
miR-708-5p	0.57	7.48E-03	miR-99a-5p	-0.54	1.15E-03
miR-99b-3p	0.56	7.83E-05	miR-181a-5p	-0.55	2.13E-04
miR-199a-5p	0.55	1.06E-02	miR-3200-3p	-0.56	5.94E-07
miR-140-5p	0.54	7.87E-03	miR-208a-5p	-0.57	4.37E-04
miR-346	0.53	9.06E-03	miR-3605-3p	-0.58	1.06E-04
miR-363-3p	0.53	2.94E-05	miR-455-5p	-0.60	2.46E-05
miR-424-3p	0.50	1.47E-04	miR-181c-5p	-0.60	1.26E-07
miR-199a-3p	0.48	5.46E-06	miR-204-5p	-0.62	4.57E-03
miR-199b-3p	0.48	2.52E-03	miR-145-3p	-0.63	2.73E-04
miR-19b-3p	0.47	1.01E-04	let-7a-3p	-0.63	1.05E-03
miR-128-3p	0.47	6.97E-05	miR-208a-3p	-0.64	1.10E-02
miR-132-3p	0.47	7.24E-05	miR-628-3p	-0.66	2.29E-05
miR-212-3p	0.46	1.15E-02	miR-148a-5p	-0.68	1.17E-02
miR-1307-5p	0.43	3.88E-02	miR-340-3p	-0.70	4.31E-07
miR-20a-5p	0.42	6.62E-05	miR-425-3p	-0.72	1.19E-03
miR-183-5p	0.41	3.61E-02	let-7g-5p	-0.74	1.34E-11
miR-574-3p	0.40	1.57E-02	miR-210-3p	-0.74	1.10E-08
miR-17-5p/106a-5p	0.40	5.92E-07	let-7d-3p	-0.74	8.54E-05
miR-19a-3p	0.39	1.25E-02	let-7f-5p	-0.75	7.09E-15
miR-505-3p	0.38	2.13E-02	miR-10a-5p	-0.75	6.97E-05
miR-504-5p	0.34	2.43E-02	miR-4454	-0.81	2.53E-14
miR-331-3p	0.31	4.79E-03	miR-125b-2-3p	-0.82	1.67E-10
miR-24-2-5p	0.26	2.69E-02	miR-4662a-5p	-0.85	2.56E-05
let-7b-5p	0.26	3.68E-02	miR-1226-3p	-0.88	2.49E-04
miR-130a-3p	0.22	2.33E-03	miR-184	-1.21	1.01E-04
miR-23a-3p	0.15	3.69E-03	let-7d-5p	-1.27	9.33E-17
miR-151a-3p	0.15	2.65E-02	miR-98-5p	-1.33	1.60E-20
miR-28-5p	-0.20	4.79E-03	miR-146a-5p	-2.04	2.22E-07
miR-181a-3p	-0.21	2.06E-02			

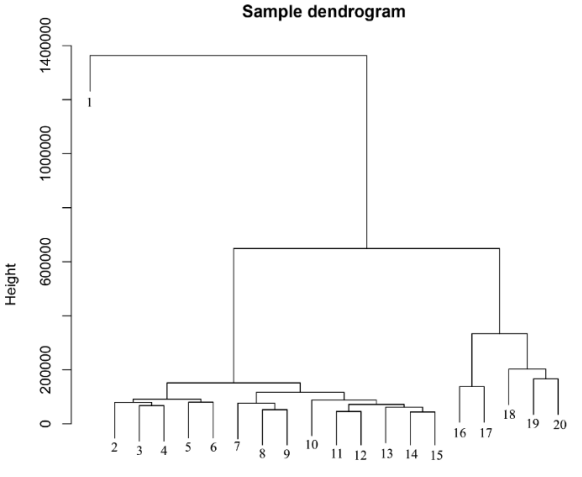
The table is organized based on decreasing log2 fold-change values. miRNA: microRNA, log2 FC: log2 fold-change. The hsa-prefix and uncorrected *p*-values were removed due to space constraints. The original format can be found here: https://www.frontiersin.org/articles/10.3389/fgene.2019.01042/full#supplementary-material

Supplementary Table S3. Differentially expressed miRNAs in PTSD subjects vs resilient subjects with FDR adjusted *p*-values <0.05.

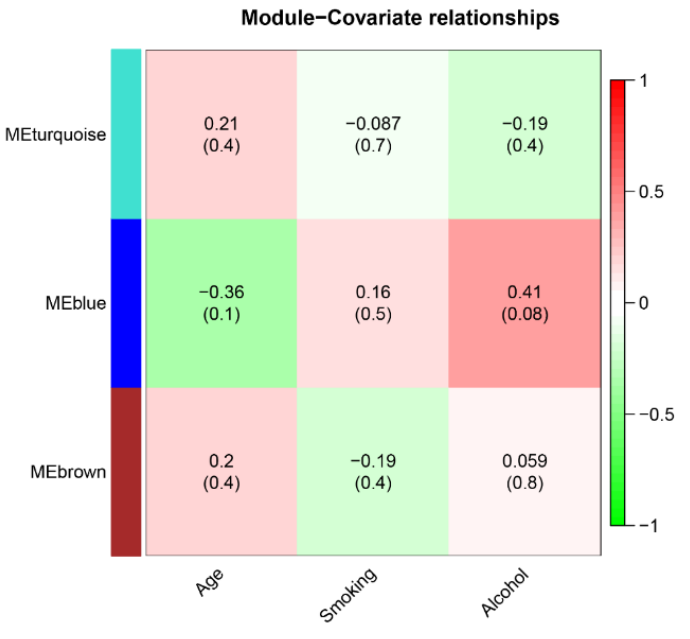
miRNA	***Log2 FC***	**P-*value***	***FDR adj* P-*value***
hsa-miR-210-3p	-0.54	2.57E-04	3.05E-02
hsa-miR-4286	-0.54	6.82E-04	4.40E-02
hsa-miR-4454	-0.61	3.94E-07	1.02E-04
hsa-miR-1246	-1.06	3.54E-04	3.05E-02

The table is organized based on decreasing log2 fold-change values. miRNA: microRNA, log2 FC: log2 fold-change.

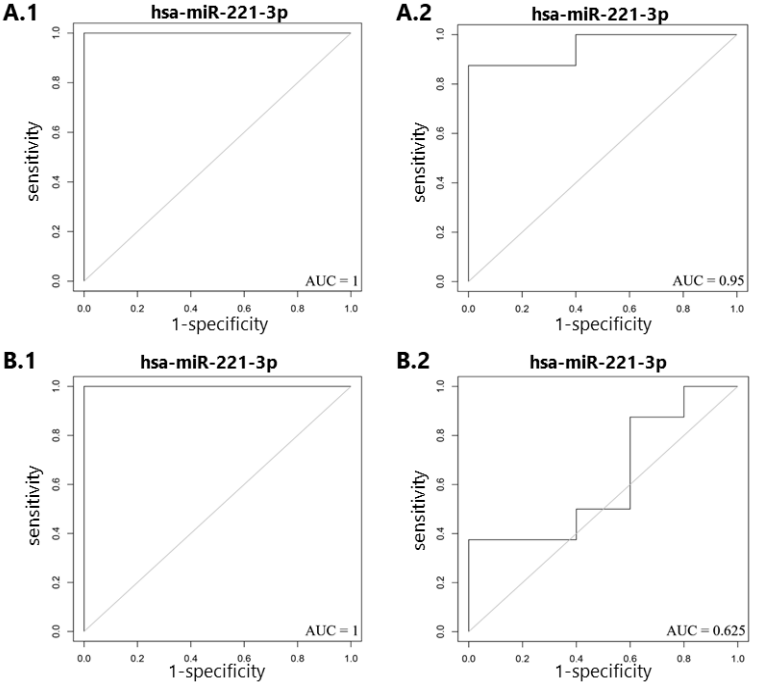
Supplementary Table S4 can be found here:
https://www.frontiersin.org/articles/10.3389/fgene.2019.01042/full#supplementary-material



Supplementary Figure S1. Sample dendrogram to detect outliers. Clustering was based on miRNA expression data. Sample names were labeled as 1-20.



Supplementary Figure S2. Correlations of modules detected by WGCNA and the following potential covariates: age, smoking status and alcohol use. *P*-values are presented between brackets. ME: module eigengene.



Supplementary Figure S3. Receiver operating characteristic (ROC) curves for the miRNA with the highest module membership in the blue module, miR-221-3p. The graphs represent PTSD vs control without confounders (A.1) or with confounders (B.1), and PTSD vs resilient without confounders (A.2) or with confounders (B.2).

Supplementary Table S5. Gene ontology (GO) biological process analysis of the target genes (N=146) of the co-expressed miRNAs from the blue module (N=79).

GO term **GO ID**	***P*-value** ***Genes***	**FDR adj** ***p*-value**
Intrinsic apoptotic signaling pathway in response to DNA damage GO:0008630	5.11E-09 *BCL2L11, BCL2A1, BCL2, E2F1, BAX, BRCA1, SIRT1, TNF, BCL2L2*	8.60E-06
Cellular response to hypoxia GO:0071456	1.19E-07 *SFRP1, IRAK1, BNIP3, PTEN, BCL2, E2F1, PMAIP1, AKT1, PTGS2, SIRT1*	2.00E-04
Cellular response to mechanical stimulus GO:0071260	1.46E-07 *GJA1, BAG3, BNIP3, AKT1, PTGS2, SLC38A2, MAP3K14, EGFR, NFKB1*	2.45E-04
Activation of cysteine-type endopeptidase activity involved in apoptotic process GO:0006919	4.96E-07 *ACER2, EGLN3, BCL2L11, SMAD3, BAX, PMAIP1, PPARG, TNF, SENP1*	8.35E-04
Negative regulation of apoptotic process GO:0043066	1.54E-06 *PRNP, SMAD3, BCL2A1, BNIP3, PTEN, SIRT1, EGFR, NFKB1, SFRP1, IRAK1, BAG3, MYC, BCL2, FLNA, AKT1, SQSTM1, BCL2L2*	2.59E-03
Positive regulation of transcription from RNA polymerase II promoter GO:0045944	2.35E-06 *RB1, BMPR2, UHRF1, BRCA1, ETS1, TNF, EGFR, PPP3R1, MYC, E2F1, AKT1, MTA2, SMAD2, SMAD4, SMAD3, CKAP2, ETV1, SIRT1, NFKB1, SENP1, ZEB2, AGO1, PPARG, MET, SQSTM1*	3.95E-03
Negative regulation of cell growth GO:0030308	8.64E-06 *SFRP1, GJA1, SMAD4, BMPR2, SMAD3, SERTAD2, BCL2, PPARG, SIRT1*	1.45E-02
Extrinsic apoptotic signaling pathway in absence of ligand GO:0097192	9.20E-06 *BCL2L11, BCL2A1, BAG3, BCL2, BAX, BCL2L2*	1.55E-02

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S6. Gene ontology (GO) molecular function analysis of the target genes (N=146) of the co-expressed miRNAs from the blue module (N=79).

GO term	***P*-value**	**FDR adj**
GO ID	***Genes***	***p*-value**
Protein binding	8.15E-13	1.13E-09
GO:0005515	*RB1, EIF4A2, TRRAP, IRS4, ADARB1, ETS1, TNF, ACTG1, GJA1, MYC, AKT2, STMN1, AKT1, KPNA2, EPHA4, RPL21, THOC2, MORF4L1, SFRP1, RRAGD, RPL26, SQSTM1, DDX6, POM121, KMT2C, PIK3R3, DNAJC27, ATP5B, PPP3R1, BCL2L11, IRAK1, BAG3, UBR5, VTI1A, PMAIP1, TBC1D17, SLC38A2, ABCA1, SMAD2, TRAP1, PRNP, SMAD4, SMAD3, MUC17, CBX1, LSM4, NFKB1, QKI, ZBTB9, CNOT1, FASN, AGO1, MKRN1, BCL2, GNAS, UBE2O, MAP3K14, BCL2L2, CDKN1C, LIN54, BMPR2, GDI2, FAF2, PTEN, BRCA1, SYNE3, MECP2, PPP6C, FAM102A, CSRP2, VCPKMT, SPTAN1, TMED5, BTN3A1, MMP2, ETV1, SIRT1, SENP1, ZEB2, RASA1, KIT, TUT1, PPARG, MET, SSX2IP, RAPGEF4, SF3B2, BCL2A1, UHRF1, NTN4, PBXIP1, PTGS2, EGFR, HIST2H4B, E2F1, FLNA, E2F2, RPS2, MTA2, PAIP2, SPEN, MAP3K3, EGLN3, GABBR1, BNIP3, ERAP1, IMMT, SSRP1, EIF3I, BAX, NCAPD2, HSPA1B, EIF3B*	
Poly(A) RNA binding	3.81E-07	5.29E-04
GO:0044822	*EIF4A2, DDX6, SF3B2, KMT2C, GDI2, ADARB1, MECP2, HIST2H4B, FLNA, RPS2, KPNA2, GCN1, SRRM2, SPEN, TRAP1, RPL21, DDX56, IMMT, SSRP1, LSM4, UTP18, QKI, CNOT1, FASN, AGO1, MKRN1, UBE2O, RPL26*	
Transcription factor binding	3.77E-06	5.23E-03
GO:0008134	*RB1, SMAD2, SMAD3, ETS1, SIRT1, NFKB1, MECP2, MYC, E2F1, BCL2, FLNA, E2F2, PPARG*	
Identical protein binding	1.11E-05	1.54E-02
GO:0042802	*RB1, PRNP, EPHA4, SMAD4, SMAD3, UHRF1, BNIP3, PTEN, ETS1, SIRT1, TNF, EGFR, NFKB1, ACTG1, SFRP1, BCL2, BAX, AKT1, PPARG, SQSTM1*	
Ubiquitin protein ligase binding	2.41E-05	3.34E-02
GO:0031625	*RB1, SMAD2, GPI, SMAD3, FAF2, BCL2, UBE2O, BRCA1, SQSTM1, EGFR, HSPA1B, ACTG1*	

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S7. Gene ontology (GO) cellular component analysis of the target genes (N=146) of the co-expressed miRNAs from the blue module (N=79).

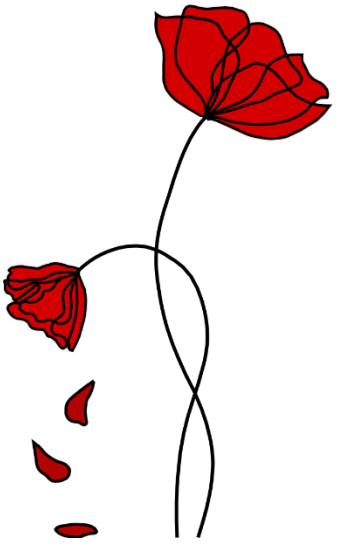
GO term **GO ID**	***P*-value** ***Genes***	**FDR adj *p*-value**
Cytosol GO:0005829	6.27E-12	8.32E-09
	EIF4A2, GPI, GDI2, IRS4, PTEN, ACTG1, MECP2, PPP6C, GJA1, MYC, AKT2, STMN1, AKT1, KPNA2, SPTAN1, RPL21, SFRP1, DDAH1, RASA1, RRAGD, PPARG, RPL26, SQSTM1, RAPGEF4, DDX6, COPA, PIK3R3, PBXIP1, RPS4Y1, ARHGAP12, PPP3R1, BCL2L11, IRAK1, BAG3, VTI1A, FLNA, PMAIP1, RPS2, TBC1D17, SMAD2, MAP3K3, EGLN3, SMAD4, SMAD3, ERAP1, LSM4, NFKB1, CNOT1, FASN, AGO1, EIF3I, BCL2, GNAS, UBE2O, PNPLA4, BAX, POLR3G, NCAPD2, MAP3K14, BCL2L2, HSPA1B, EIF3B	
Nucleoplasm GO:0005654	8.76E-08	1.16E-04
	RB1, GPI, LIN54, SF3B2, TRRAP, KMT2C, PTEN, BRCA1, ADARB1, RPS4Y1, ETS1, PPP3R1, HIST2H4B, MYC, AKT2, EPB41L2, UBR5, TCEAL1, E2F1, E2F2, AKT1, RPS2, MTA2, KPNA2, SRRM2, SPEN, SMAD2, TRAP1, EGLN3, SMAD4, SMAD3, BNIP3, CBX1, SSRP1, THOC2, LSM4, UTP18, SIRT1, NFKB1, SENP1, MORF4L1, AGO1, POLR3G, PPARG, NCAPD2, SQSTM1, HSPA1B, EIF3B	
Mitochondrial outer membrane GO:0005741	2.53E-06	3.36E-03
	PRNP, EPHA4, GJA1, BCL2L11, BCL2A1, BNIP3, BCL2, BAX, PMAIP1, BCL2L2	
Nucleus GO:0005634	1.73E-05	2.29E-02
	CDKN1C, RB1, TRRAP, PTEN, BRCA1, ADARB1, ETS1, ACTG1, MECP2, CSRP2, MYC, AKT2, ZNF800, AKT1, KPNA2, MMP2, ETV1, UTP18, SIRT1, SENP1, MORF4L1, ZEB2, RRAGD, TUT1, PPARG, SSX2IP, ZNF597, DDX6, UHRF1, KMT2C, PBXIP1, RPS4Y1, PTGS2, DNAJC27, EGFR, ATP5B, HIST2H4B, ZNF629, IRAK1, SERTAD2, EPB41L2, UBR5, TCEAL1, E2F1, FLNA, PMAIP1, RPS2, SPEN, SMAD2, PRNP, EGLN3, SMAD4, SMAD3, BNIP3, CBX1, SSRP1, NFKB1, QKI, ZBTB9, CNOT1, AGO1, BCL2, GNAS, UBE2O, BAX, NCAPD2, BRWD1	
Membrane GO:0016020	2.49E-05	3.31E-02
	GPI, DDX6, COPA, GDI2, RPS4Y1, IER3IP1, TNF, SYNE3, EGFR, ACTG1, ATP5B, HIST2H4B, STMN1, UBR5, FLNA, RPS2, MTA2, KPNA2, SPTAN1, AGFG2, GCN1, TRAP1, RPL21, DDX56, ERAP1, IMMT, CNOT1, FASN, KIT, BCL2, GNAS, BAX, PNPLA4, NCAPD2, RPL26, RAPGEF4	
Mitochondrion GO:0005739	3.34E-05	4.43E-02
	DDX6, PTEN, CROT, DNAJC27, MECP2, ATP5B, GJA1, PPP3R1, BCL2L11, MYC, E2F1, AKT1, PMAIP1, TRAP1, PRNP, BNIP3, MMP2, IMMT, SIRT1, NFKB1, DDAH1, FASN, BCL2, BAX, PNPLA4, HSPA1B	

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

CHAPTER 5

Isolating and analyzing plasma neuron-derived exosome microRNAs: a working protocol

Clara Snijders



Abstract

The discovery that peripherally accessible exosomes could potentially yield valuable information regarding (patho)physiological mechanisms occurring within the brain, resulted in a steep increase in the number of studies aiming to use the content of these exosomes as biomarkers for neurological disorders. This requires validated procedures to isolate exosomes, and to extract and analyze their content. Here, we present a protocol for isolating and sequencing the miRNA content of so-called plasma neuron-derived exosomes, i.e. exosomes secreted by neurons, which can be found within blood plasma. In doing so, we aim to start standardizing the field of exosome isolation and miRNA analysis.

Keywords: exosomes, neuron-derived exosomes, microRNA, methodology, blood

Introduction

Exosomes are a class of extracellular vesicles with a diameter of ~40-150nm which are released from multivesicular bodies (MVBs) after fusion with the plasma membrane [1]. Specifically, inward budding of intracellular endosomal membranes leads to MVBs carrying intraluminal vesicles [2]. Fusion of the MVBs with the plasma membrane then releases these vesicles in the extracellular space in the form of exosomes. Exosomes have been found to carry a variety of molecules such as proteins, nucleic acids (including microRNAs), lipids and metabolites, which they transfer and deliver to recipient cells [3, 4]. This ability to carry their content to distant cells makes them potentially interesting tools for drug delivery [5]. Simultaneously, since the encapsulated (nucleic acid) material is protected from degradation and is therefore relatively stable, and since exosomes are present in most easily accessible biofluids such as blood [6], urine [7] and saliva [8], these extracellular vesicles are increasingly being recognized as interesting biomarker candidates [9].

The discoveries that exosomes cross the blood-brain barrier [10] and carry cell-type specific markers on their membrane [11], led to an ongoing increase in the number of studies aiming to isolate so-called blood-based neuron-derived exosomes (NDEs), i.e. brain-derived exosomes which are present in blood [12]. Analyzing their content is particularly interesting to indirectly gain insights into (patho)physiological mechanisms occurring within the brain, especially since exosomes have been found to be involved in a variety of central processes such as myelin maintenance [13], synaptic plasticity [14], and neuronal and axonal trophic support [15]. As such, it could be highly valuable to assess their potential implication in several neurological and neurodegenerative disorders along with their usefulness to serve as minimally invasive, yet highly relevant, biomarkers [12].

Currently, there is no clear consensus on how NDEs should best be isolated and analyzed. Moreover, assessing their microRNA (miRNA) content is even less well explored. While several (pilot) studies have shown proof of concept for analyzing the protein content of blood exosomes enriched for neuronal origin [16-19], only one study explored the miRNA content of human blood NDEs using miRNA-specific qPCRs [20]. Therefore, the present methodological chapter presents the result of a year of optimizing several isolation and analysis techniques, and aims to start standardizing critical steps in the isolation of NDE miRNAs from limited amounts of human plasma. The protocol presented below consists of isolating total exosomes using a commercially available precipitation method

(ExoQuick, System Biosciences), followed by immunoprecipitation using biotinylated antibodies against L1CAM (CD171), which is a surface marker known to be enriched in neuronal tissue. A similar method was developed and published before, and shows that several markers believed to be relatively specific to neurons (e.g. MAP2, NCAM, NFL, and neuron-specific enolase) are enriched in the recovered L1CAM+ exosomes and are present in greater amounts than in total plasma exosome fractions [12]. Here, the miRNA content of these vesicles is then isolated using an adapted version of the miRNeasy Micro Kit (Qiagen) protocol and prepared for next generation sequencing using the NEBNext Multiplex Small RNA Library Prep set (New England BioLabs) with several modifications, as detailed below (Figure 1). This protocol was then applied to plasma, serum and urine samples, the results of which are presented in the next chapter. Researchers are now encouraged to apply the present protocol to their own samples in order to compare obtained fractions in terms of exosome, miRNA and DNA library yield.

Materials

Isolation of exosomes and enrichment for neuronal origin

- ExoQuick™ Plasma Prep with Thrombin (System Biosciences)
- Basic Exo-Flow™ Capture Kit (System Biosciences)
- CD171 monoclonal antibody 5G3 coupled to Biotin (ThermoFisher)
- Halt™ Protease Inhibitor Cocktail (ThermoFisher, or equivalent)
- Magnet to capture magnetic beads

Isolation of total (including small) RNA

- miRNeasy Micro Kit (Qiagen)
- 100% and 80% ethanol (etOH)
- Chloroform without added isoamyl alcohol

Library preparation for next generation sequencing

- NEBNext® Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs)
- 20% polyethylene glycol (PEG)/2.5 M NaCl solution
- Sera-Mag Speedbeads™ (GE Healthcare UK Unlimited)
- Novex™ Hi-Density TBE Sample Buffer (5X) (ThermoFisher)
- 10x Tris-Borate-EDTA (TBE) (BioPioneer)
- Novex™ TBE 10% gel (ThermoFisher)
- DNAmark™ 20bp DNA ladder (G Biosciences)
- SYBR™ Gold Nucleic Acid Gel Stain (ThermoFisher)

- ChIP DNA Clean and Concentrator™ kit (Zymo Research)
- Magnet to capture magnetic beads
- 22 gauge needle
- 80% etOH
- Qubit™ assay tubes (ThermoFisher)
- Gel elution buffer: 0.5 M LiCl, 0.1% SDS, 5 mM EDTA, 10 mM Tris pH 7.5
- TET buffer : 10 mM Tris 8.0, 0.1 mM EDTA, 0.05% Tween

Quantification of RNA and cDNA libraries

- Agilent RNA 6000 Nano Kit (Agilent Technologies)
- Agilent High Sensitivity (HS) DNA kit (Agilent Technologies)
- Qubit™ dsDNA HS Assay Kit (ThermoFisher)
- Qubit™ assay tubes (ThermoFisher)

Methods

Isolation of exosomes and enrichment for neuronal origin

The isolation of exosomes is done using the ExoQuick Plasma Prep with Thrombin (System Biosciences). The protocols provided by System Biosciences can be followed. Modifications and additions are indicated in bold:

- **Thaw 850 µl plasma**.
- Spin for 15 min at 3000x g (4°C).
- Recover the supernatant (~800 µl) and add 6 µl of Thrombin provided with the kit (final concentration 5 U/ml).
- Incubate for 5 min while inverting the samples (room temperature; RT)
- Spin for 5 min at 11000x g (4°C).
- Recover the supernatant (~750 µl) and add **200 µl of ExoQuick.**
- Flick and invert the tubes several times, and **incubate for 2 h (4°C).**
- **Spin the samples for 1 h** at 1500x g (4°C).
- Remove the supernatant, and **re-suspend the exosome fractions in 100 µl 1x PBS** supplied with protease inhibitors.
- Keep the fractions on ice.
- In the meantime, prepare the magnetic beads for immunoprecipitation:
    - » Pipette 40 µl of beads into 1.5 ml tubes (prepare 1 tube per sample).
    - » Place the tubes on the magnetic stand.

    - » After 2 min, remove the supernatant and wash the beads twice by adding 500 µl of Bead Wash Buffer.
    - » Remove the tubes from the magnetic stand and **add 4 µg of the CD171 monoclonal antibody 5G3 couples to Biotin.**
    - » **Incubate the samples on ice for 3 h,** and gently flick the tubes ever 30 min.
    - » Add 200 µl of Bead Wash Buffer and mix gently.
    - » Place the tubes on the magnetic stand.
    - » After 2 min, remove the supernatant and wash the beads twice by adding 500 µl of Bead Wash Buffer.
    - » Remove the tubes from the magnetic stand and re-suspend the antibody-beads fractions in 400 µl of Bead Wash Buffer.
- **Add the entire volume of total exosome fractions (100 µl) to the beads (400 µl).**
- Let the samples rotate overnight for capture (at least 16 h at 4°C using a low rotation speed).
- Place the samples on the magnetic stand.
- After 2 min, remove the supernatant and wash the samples twice by very carefully adding 500 µl Bead Wash Buffer.
- Remove the tubes from the magnetic stand and **add 250 µl of Exosome Elution Buffer.**
- Flick the tubes to mix, and incubate the samples on a shaker at RT for 2 h.
- Place the samples on the magnetic stand.
- After 2 min, transfer the supernatants containing the eluted NDEs to a fresh tube.
- **Immediately proceed with the RNA isolation.**

Isolation of (small) RNA

The isolation of (small) RNAs is done using the miRNeasy Micro Kit (Qiagen). Follow the protocol provided by Qiagen and start at the step of QIAzol Lysis Reagent addition. Modifications and additions to the protocol are indicated in bold:

- To 250 µl of freshly isolated NDEs, **add 1000 µl of Qiazol lysis buffer** and vortex for 15 s.
- Incubate for 5 min (RT).
- **Add 250 µl of chloroform and shake vigorously for 1 min.**
- **Incubate for 10 min (RT).**
- Spin for 15 min at 12000x g (4°C).

- Transfer the upper aqueous phase (~700 µl) to a new tube and add 1.5 volumes of 100% etOH (~1050 µl).
- Invert the tube several times, and add the sample to a column provided with the kit in steps of 700 µl.
- **Spin for 15 s at 1000x g (RT).** Repeat this step along with the previous step until the whole sample passed through the column.
- Add 700 µl of buffer RWT and **spin for 15 s at 8500x g (RT).**
- Add 500 µl of buffer RPE and **spin for 15 s at 8500x g (RT).**
- Add 500 µl of freshly prepared 80% etOH and spin for 2 min at 8500x g (RT).
- Place the column in a new collection tube and spin for 5 min at full speed (~20000x g, RT) with open caps to dry the membrane.
- Place the column in a new 1.5 ml tube and add 14 µl of water to the membrane.
- Incubate for 2 min (RT).
- **Spin for 1 min at 100x g, then for 1 min at 16000x g (both at RT).**
- **Keep 1 µl for Agilent Bioanalyzer analysis, and immediately freeze the remaining volume at -80°C.**
    - » Note: expect small RNA concentrations around 4 ng/µl and ~80% of miRNA content (by default defined as fragments sizes of 10-40 nucleotides).

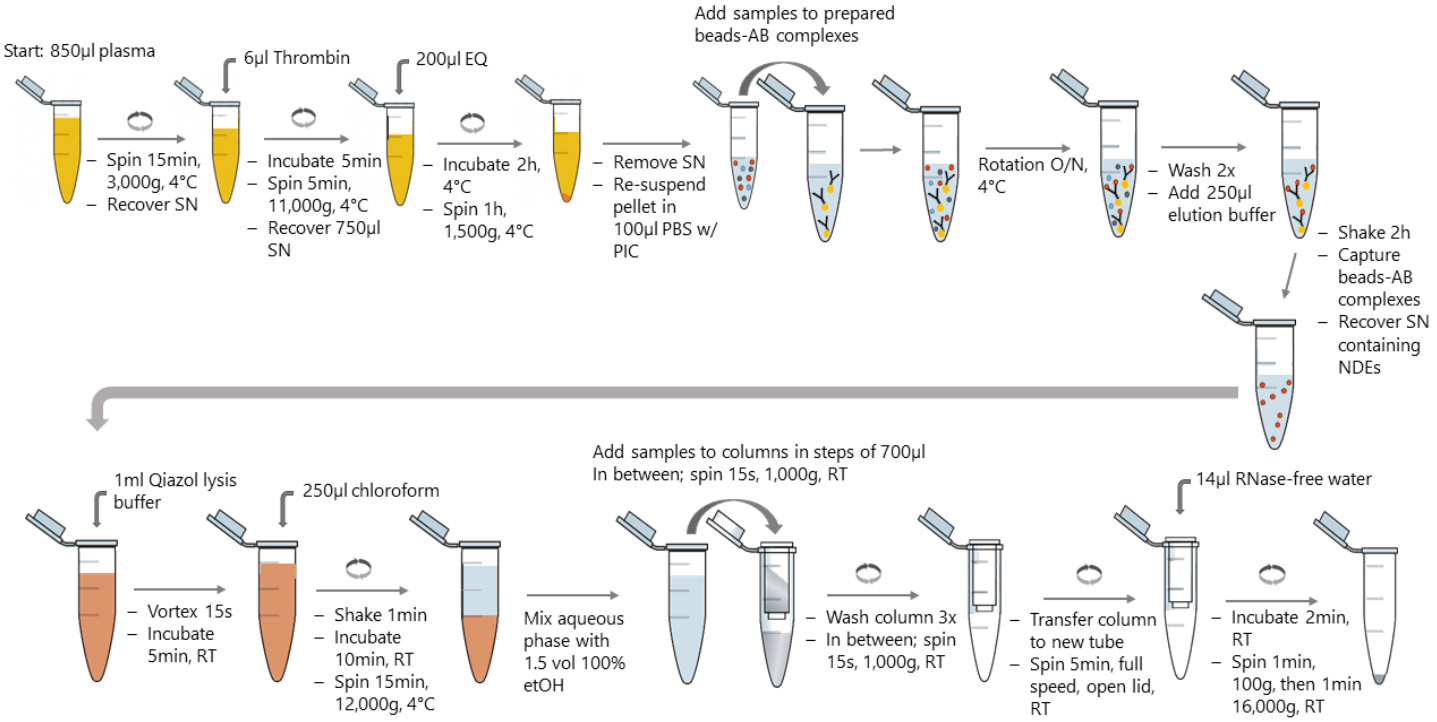


Figure 1. Overview of the workflow to isolate total RNA (including small RNAs) from neuron-derived exosomes. Double-sided circle arrows reflect spinning steps. SN: supernatant, EQ: ExoQuick, w/: with, PIC: protease inhibitor cocktail, O/N: overnight, AB: antibody, NDEs: neuron-derived exosomes, vol: volumes.

Library preparation for next generation sequencing

The library preparation is done using the NEBNext® Multiplex small RNA library prep kit (NEB). Follow the protocol provided by NEB, starting with 6.5 µl of RNA and using half the amount of adaptors, as specified by the protocol recommendations. Given the length and complexity of the protocol, and since no modifications were made in the first part of the protocol, the protocol below starts at the end of the polymerase chain reaction (PCR) step. Modifications and additions are indicated in bold:

- **Following reverse transcription and PCR, store the samples overnight at -20°C.**
- **Thaw the PCR product (100 µl) and to each sample, add 100 µl of cold 20 % PEG/2.5 M NaCl and 3 µl of magnetic Speedbeads.**
- **Incubate for 10 min (RT).**
- **Place the samples on the magnetic stand.**
- **Once clear, remove the supernatant and wash the samples twice by adding 180 µl of 80% etOH.**
- **Once clear, remove the supernatant and let the beads dry for ~5 min (RT).**
- **Add 15 µl of 1x sample elution buffer, vortex well and incubate for at least 5 min (RT).**
- Run the samples (15 µl) and the ladder (1.5 µl) on a 10% TBE gel for ~75 min at 200V.
  - » Note: use the dyes of the sample buffer as a size indication; on a 10% TBE gel, Bromophenol Blue (dark blue) will run at the same speed as DNA fragments of 35 base pairs (bp) in size, while Xylene Cyanol (blue/green) will run with DNA fragment sizes of 120 bp (see further clarifications on the ThermoFisher website under Novex gels).
- Transfer the gel to a gel holder and incubate with 3 µl of SYBR Gold for 5 min.
- Visualize the gel under UV light.
- Cut out the gel bands corresponding to 140-160 bp, and transfer the gel fragments to perforated Qubit tubes placed within 1.5 ml tubes.
- Spin the samples for 30 s at 10000x g (RT), or until the gel is crushed and passed through the Qubit tube.
- Remove the Qubit tubes, **add 150 µl of gel elution buffer to the gel fragments and shake the samples for 3 h (RT).**
- Clean the DNA using the ChIP DNA clean and concentrator kit (RT):
  - » Add 750 µl of ChIP DNA Binding Buffer and mix by pipetting up and down.
  - » Transfer the total volume to a provided column and spin for 30 s at 10000x g.
  - » Discard the flow-through.

- » Add 200 µl of Wash Buffer, spin for 30 s at 10000x g.
- » Discard the flow-through and repeat the washing step once more.
- » Place the column in a new tube.
- » **Add 15 µl TET buffer** and spin for 30 sat 10000x g.

– Verify the size of your libraries on a Bioanalyzer using a HS DNA chip and measure the DNA concentration by Qubit (HS DNA kit).
  - » Note: on the Bioanalyzer, expect a small peak around 150 bp. On the Qubit, expect stock concentrations ranging from 0.3 ng/µl to 1 ng/µl.

Small RNA-sequencing and differential expression analysis

Samples with different unique barcodes can be pooled and sequenced together in one multiplexed run. After performing the quality control using FastQC [21], the data needs to be processed using the miRDeep2 software for 3′ adapter trimming, size selection (only retain sequences ≥ 18 nucleotides long), and mapping to the miRBase miRNAs. The set of reads is then used as input to the DESeq package in R for the detection of differentially expressed miRNAs. To account for bias introduced by miRNAs with very low abundance, only those miRNAs with a minimum of 10 read counts across samples (on average) are retained for further analyses.

References

1. van Niel, G., G. D'Angelo, and G. Raposo, *Shedding light on the cell biology of extracellular vesicles*. Nat Rev Mol Cell Biol, 2018. **19**(4): p. 213-228.
2. Stoorvogel, W., et al., *The biogenesis and functions of exosomes*. Traffic, 2002. **3**(5): p. 321-30.
3. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells*. Nat Cell Biol, 2007. **9**(6): p. 654-9.
4. Zhang, Y., et al., *Exosomes: biogenesis, biologic function and clinical potential*. Cell & Bioscience, 2019. **9**(1): p. 19.
5. Bunggulawa, E.J., et al., *Recent advancements in the use of exosomes as drug delivery systems*. Journal of Nanobiotechnology, 2018. **16**(1): p. 81.
6. Cheng, L., et al., *Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood*. J Extracell Vesicles, 2014. **3**.
7. Nilsson, J., et al., *Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer*. Br J Cancer, 2009. **100**(10): p. 1603-7.
8. Zlotogorski-Hurvitz, A., et al., *Human saliva-derived exosomes: comparing methods of isolation*. J Histochem Cytochem, 2015. **63**(3): p. 181-9.
9. Lin, J., et al., *Exosomes: novel biomarkers for clinical diagnosis*. ScientificWorldJournal, 2015. **2015**: p. 657086.
10. Alvarez-Erviti, L., et al., *Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes*. Nat Biotechnol, 2011. **29**(4): p. 341-5.
11. Wu, D., et al., *Profiling surface proteins on individual exosomes using a proximity barcoding assay*. Nature Communications, 2019. **10**(1): p. 3854.
12. Mustapic, M., et al., *Plasma Extracellular Vesicles Enriched for Neuronal Origin: A Potential Window into Brain Pathologic Processes*. Front Neurosci, 2017. **11**: p. 278.
13. Domingues, H.S., et al., *Exosome Circuitry During (De)(Re)Myelination of the Central Nervous System*. Front Cell Dev Biol, 2020. **8**: p. 483.
14. Bahrini, I., et al., *Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia*. Scientific Reports, 2015. **5**(1): p. 7989.
15. Kramer-Albers, E.M., et al., *Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons?* Proteomics Clin Appl, 2007. **1**(11): p. 1446-61.
16. Abner, E.L., et al., *Plasma neuronal exosomal levels of Alzheimer's disease biomarkers in normal aging*. Ann Clin Transl Neurol, 2016. **3**(5): p. 399-403.
17. Fiandaca, M.S., et al., *Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study*. Alzheimers Dement, 2015. **11**(6): p. 600-7 e1.
18. Goetzl, L., N. Darbinian, and E.J. Goetzl, *Novel window on early human neurodevelopment via fetal exosomes in maternal blood*. Ann Clin Transl Neurol, 2016. **3**(5): p. 381-5.
19. Winston, C.N., et al., *Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome*

protein profile. Alzheimers Dement (Amst), 2016. **3**: p. 63-72.

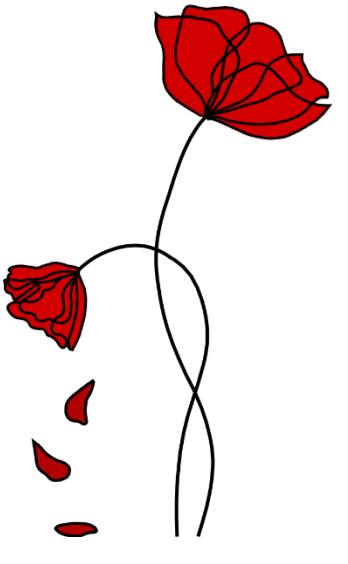
20. Cha, D.J., et al., *miR-212 and miR-132 Are Downregulated in Neurally Derived Plasma Exosomes of Alzheimer's Patients*. Front Neurosci, 2019. **13**: p. 1208.

21. Andrews, S. *FastQC A Quality Control Tool for High Throughput Sequence Data*. Available from: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

CHAPTER 6

Profiling microRNAs from blood and urine neuron-derived exosomes in subjects with combat-related post-traumatic stress disorder

Clara Snijders, Laurence de Nijs, Julian Krauskopf, Charisse Winston, Ehsan Pishva, Daniel van den Hove, Gunter Kenis, Myeong Ok Kim, Caroline Nievergelt, Victoria Risbrough, Bart Rutten



Abstract

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that can develop upon exposure to a shocking event. These last years, several microRNAs (miRNAs) present in peripheral biofluids such as blood plasma have been found associated with the development of PTSD. The discovery that blood also contains exosomes that originate from the brain, opened up new possibilities to indirectly gain insights into (patho)physiological processes occurring within the central nervous system in a relatively non-invasive manner. In this feasibility study, we aimed to profile miRNAs from limited amounts of plasma neuron-derived exosomes (NDEs) using high throughput sequencing, and explored whether these could serve as markers for combat-related PTSD. Differential expression analysis revealed higher levels of two miRNAs, miR-581-5p and miR-542-3p, in NDEs from individuals with PTSD (N=6) as compared to trauma-exposed healthy age- and gender-matched controls (N=6). We then assessed whether we could obtain similar results when applying the same methodology to (i) urine samples belonging to the same individuals, and (ii) 10+ year old serum samples belonging to a Dutch military cohort. Our main preliminary findings suggest that (i) plasma NDEs can be used to profile miRNAs, (ii) the differential expression of miR-581-5p and miR-542-3p could be of interest for replication by future PTSD studies using larger sample sizes, and (iii) sample age seems to negatively impact the RNA content of NDEs. Future studies using larger sample sizes are warranted in order to replicate our plasma findings, and explore the usefulness of urine NDEs.

Keywords: post-traumatic stress disorder, neuron-derived exosomes, microRNAs, plasma

Introduction

Post-traumatic stress disorder (PTSD) is a mental health disorder that can develop following exposure to a (potentially) traumatic event [1]. Given the severe impact of PTSD on well-being and the time window between the trauma exposure itself and the onset of PTSD, there is a great need and opportunity for early identification of affected individuals [1, 2]. Moreover, identifying diagnostic markers of combat-related PTSD specifically would be highly relevant, for example for those military members who, upon completion of their military service, might minimize their symptoms in order to avoid any potential stigma- or duty-related consequences, or are not capable of fully disclosing the extent of their symptoms [3-5].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression, primarily by degrading target messenger RNAs (mRNAs) or by repressing their translation [6]. Within the central nervous system (CNS), specific miRNAs have repeatedly been found involved in synaptic plasticity [7-9], learning, and the formation of stress-associated memories [10-12]. Within peripheral biofluids, freely circulating miRNAs are remarkably stable due to being bound to Argonaute2 (Ago2) [13] or to high- or low-density lipoproteins [14]. Over the last years, circulating miRNAs have been suggested to serve as potentially suitable biomarker candidates for several disorders, including PTSD [15]. However, previous studies have suggested that the majority of protein-bound miRNAs could be by-products of cell injury or cell death and their exact functions remain unexplored [16]. Therefore, it has been suggested that miRNAs encapsulated within extracellular vesicles such as exosomes could be better suited for sampling and analysis [16, 17].

Exosomes are small vesicles (40-150nm) present in several body fluids including blood [18], cerebrospinal fluid (CSF) [19] and urine [20], and have an endosomal origin [21]. Endosomes formed within a cell are packed together within multivesicular bodies (MVBs) that fuse with the plasma membrane to release their content as exosomes. Recently, exosomes have received increasing attention, particularly since they were found to transfer proteins and different RNA molecules, including miRNAs, between cells [22, 23]. Moreover, exosomes carry specific surface proteins based on their tissue of origin [24]. In order to indirectly gain insights into (patho)physiological processes occurring within the brain, several studies have isolated and analyzed blood-based neuron-derived exosomes (NDEs) using antibodies directed against the neural cell adhesion molecule L1 (L1CAM) [25-29]. Given that PTSD is mediated by functional and structural aberrations

within the brain, and given the many implications of miRNAs in key processes within the CNS, exploring the miRNA content of NDEs could be of great added value in order to gain insights into the roles of miRNAs within the CNS of individuals with PTSD. This could then lead the way to assessing their potential usefulness as relatively easily accessible biomarkers of disease.

In this proof-of-concept study, the aim was to identify miRNA expression profiles in plasma NDEs from individuals with PTSD. This was done in an agnostic manner through sequencing NDE-derived miRNAs from subjects with PTSD and healthy controls who were exposed to similar levels of trauma. Two additional objectives were to (i) perform a first exploration of urine NDE miRNA profiles using urine samples belonging to the same individuals and compare obtained miRNA profiles between biofluids, and (ii) apply the same methodology to 10+ year old serum samples belonging to a Dutch military cohort, and assess whether our plasma findings would replicate in this independent cohort.

Materials and methods

Participants

CESAMH

Individuals were recruited at the Center of Excellence for Stress and Mental Health (CESAMH) at the San Diego Veteran Health Administration (VA) from different converging studies or from direct enrollment to the study. For the present study, 12 participants were selected based on their severity of PTSD symptoms and levels of trauma exposure (8 males, 4 females). These subjects filled out a battery of self-report tests including the PTSD Checklist (PCL). All participants were exposed to combat-related trauma, were non-smokers and non-drinkers at the time of their visit, and consented to donating blood and urine. Whole blood was collected in EDTA tubes which were centrifuged at 1500x g for 15 min. Within 30 min following sample collection, plasma and urine samples were aliquoted in 1 and 2ml tubes, respectively, and stored at -80°C until further use. All participants provided written informed consent and HIPAA compliance. The study was approved by the Institutional Review Board of the VA San Diego Healthcare System.

PRISMO

Subjects were selected from the Prospective Research In Stress-related Military Operations (PRISMO) study (N=24, all males) [30]. Three subgroups were selected based

on the severity of trauma exposure during deployment to Afghanistan and the severity of PTSD symptoms upon return; (i) trauma-susceptible individuals who developed PTSD following combat-related trauma exposure, (ii) trauma-resilient individuals who experienced trauma but did not develop PTSD-related symptoms, and (iii) deployed but non trauma-exposed and mentally healthy control subjects. Blood samples were collected at the Utrecht University Medical Center at several time points before and after trauma exposure. The present study made use of serum samples collected at six months post-deployment, which were stored at -80°C for 10-15 years. Information on trauma exposure was collected using a 19-item deployment experiences checklist [31], and six months post-deployment PTSD symptoms were verified using the 22-item Self-Rating Inventory for PTSD (SRIP) [31, 32]. Differences in age, number of previous deployment, PTSD score at post-deployment and trauma exposure were assessed using Kruskal-Wallis tests. Pairwise comparisons were performed using the Dunn-Bonferroni approach. This study was approved by the ethical committee of the University Medical Center Utrecht (01-333/0) and conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent.

Exosome isolation and enrichment for neuronal origin

The isolation of plasma exosomes and enrichment for neuronal origin was done as described in Chapter 5. Briefly, plasma aliquots (~850 µl) were pre-treated with thrombin at a final concentration of 5 U/ml. After spinning at 11,000x g for 5 min, supernatants (750 µl) were collected and 200 µl ExoQuick Exosome Precipitation Solution (System Biosciences) was added. Urine samples (1.6 ml) received 350 µl ExoQuick-TC (System Biosciences), while serum samples (500 µl) received 135 µl ExoQuick (System Bioscinces). Blood (plasma and serum) and urine samples were kept at 4°C for 2 h or 24 h, respectively. Samples were centrifuged at 1500x g (4°C) for 1 h (blood) or 2 h (urine). Exosome pellets were re-suspended in 100 µl Phosphate-Buffered Saline (PBS) supplied with a protease inhibitor cocktail (Roche Applied Sciences). The samples were used immediately for neuronal enrichment.

The protocol supplied with the Basic Exo-Flow Capture Kit (System Biosciences) to capture NDEs was followed with minor modifications. Briefly, the streptavidin-coated magnetic beads (System Biosciences) were incubated with 4 µg of mouse anti-human CD171 (L1CAM) biotinylated antibody (clone 5G3, eBioscience) for 3 h. The exosome suspensions and the prepared beads were incubated overnight. Unbound exosomes were washed away with the supplied wash buffer, and the remaining NDEs coupled to

beads were shaken for 2 h at room temperature. The NDEs were re-suspended in 250 µl of the provided elution buffer. All samples were used immediately for RNA isolation.

RNA isolation

Exosomal RNA was isolated using the miRNeasy Micro Kit (Qiagen) with minor modifications [33]. Briefly, for each sample, 1 ml of QIAzol lysis buffer was added to 250 µl of total NDE solution. After addition of 250 µl of chloroform, the samples were spun at 12,000x g for 15 min. The aqueous phases (~700 µl) were transferred to the provided purification columns and all samples were spun at 1000x g until the whole sample passed through. The columns were washed several times and RNA was eluted in 14 µl RNase-free water. Of each sample, aliquots of 1 µl were kept for quantity determination using the Agilent Bioanalyzer 2100 with a Small RNA Chip (Agilent Technologies). Remaining samples were immediately stored at -80°C until further use.

Small RNA library preparation and next generation sequencing

For serum RNA samples, the NextFlex Small RNA-seq v3 Automation Kit with UDIs (Perkin Elmer) was used. For plasma and urine RNA sample, the NEBNext Multiplex Small RNA Library Prep Set (New England Biolabs) was used. Libraries were prepared according to the manufacturer instructions using less adapters as specified in the manufacturer's protocols. Following PCR amplification, all samples were kept at -20°C overnight. For plasma and urine samples, sample clean-up was done as explained in Chapter 5. Briefly, 2 µl of Sera-Mag SpeedBeads (GE Healthcare) and 100 µl 20% PEG 8000/2.5 M NaCl was added to each sample, and samples were washed 80% ethanol. The beads were air-dried and the DNA was eluted in 15 µl Novex Hi-Density Tris-Borate-EDTA (TBE) Sample Buffer (ThermoFisher). Following gel electrophoresis, DNA libraries were visualized, gel fragments corresponding to the size of miRNAs (140-160 base pairs) were recovered and crushed using perforated Eppendorf tubes. Final sample clean-up was done using the ChIP DNA Clean & Concentrator kit (Zymo Research) and DNA was eluted in 15 µl of Tris-EDTA buffer.

DNA concentrations were measured using a Qubit HS dsDNA kit on a Qubit fluorometer (Life Technologies). Libraries were sequenced to a depth of approximately 10 million reads on an Illumina HiSeq2500sequencing platform. After quality control using FastQC (v. 0.11.3), reads were preprocessed and mapped to the latest release of miRBase (v. 21) [34] using miRge with default settings [35].

Differential expression analysis

In order to account for bias introduced by low abundant miRNAs, only those miRNAs with an average of 10 counts or more across samples were selected for differential expression analysis. Data normalization and differential expression analysis were conducted using the DESeq2 package in R (v. 1.22.2) [36] while correcting for sex, age, and smoking. For discovery purposes, resulting *p*-values were corrected using the False Discovery Rate (FDR) method at 10% [37].

Target gene pathway and enrichment analyses

Gene targets of differentially expressed miRNAs were identified using miRTarBase 6.0 [38]. The online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 [39, 40] was then used to detect enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms.

Exosome characterization and size determination

Leftover plasma and urine samples from 3 healthy control subjects were pooled and used for exosome isolation and enrichment for neuronal origin as described above. These fractions were subsequently used for exosome characterization and size determination using Nanoparticle Tracking Analysis (NTA), Western blotting and Transmission Electron Microscopy (TEM).

Nanoparticle Tracking Analysis (NTA)

NTA was performed to characterize size distributions of both total exosome fractions and NDEs. Exosome and NDE preparations were diluted 1:2000 and 1:1000 with 1x PBS, respectively. Samples were injected into a NanoSight LM10 instrument (Malvern Instruments) and particle movements were recorded three times for 10 seconds at ambient temperature.

Western Blotting

Total plasma exosomes resuspended in PBS were lysed by adding an equal volume of RIPA buffer (ThermoFisher) supplemented with a protease inhibitor cocktail (Sigma Aldrich). Protein quantification was performed using a BCA kit (ThermoFisher) and 20µg of plasma or SH-SY5Y cell lysates were separated using a 10% SDS-polyacrylamide gel (SDS-PAGE). The proteins were transferred to nitrocellulose membranes which were blocked for 1 h in Odyssey blocking buffer (Li-Cor) in Tris-Buffered Saline (TBS) (Li-cor) or 3% Bovine Serum Albumin (BSA) in TBS-Tween 20 (TBST). Membranes were then incubated with mouse anti-CD63 (1:1000) (ab193349; Abcam), mouse anti-calnexin (1:500)

(66903-1-Ig; Proteintech), rabbit anti-TSG101 (T5701; Sigma Aldrich) or mouse anti-Alix (1:1000) (VMA00273, Biorad). All antibodies were diluted in blocking buffer with TBS or BSA and incubation was done overnight at 4°C. As a positive control for calnexin, SH-SY5Y cells were cultured, lysed using RIPA and run alongside the samples. Membranes were washed three times with 1x TBST, incubated for 1h at room temperature with a donkey anti-mouse antibody (1:10,000) (925-68072, Li-Cor), donkey anti-rabbit antibody (1:10,000) (925-32213, Li-Cor) or an HRP-conjugated anti-mouse secondary antibody (1:3,000) (HAF007, R&D Systems), and washed three times with TBS and TBST. Images were captured using an Odyssey system (Li-Cor) or an ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

Transmission electron microscopy (TEM)

Formvar-carbon-coated copper grids (100 mesh, Electron Microscopy Sciences) were placed on 20µl drops of plasma or urine total exosome fractions displayed on a Parafilm sheet. After allowing the material to adhere to the grids for 10 minutes, grids were washed 3 times by rising through 200µl drops of milli-Q water before being left for 1 min on 2% uranyl acetate (Ladd Research Industries). Excess solution was removed with Whatman 3MM blotting paper, and grids were left to dry for a few minutes before viewing. Grids were examined using a Tecnai G2 Spirit BioTWIN transmission electron microscope equipped with an Eagle 4k HS digital camera (FEI).

Results

CESAMH: Plasma neuron-derived exosomes

Demographics

A total of 12 trauma-exposed subjects were included in this pilot study. Half of them had current PTSD at their time of visit (N=6; PCL score = 50.50 ± 2.95 standard error (SE)), while the other half did not (N=6; PCL score = 5.57 ± 1.61 SE). Cases and control subjects were matched based on age (43.17 ± 6.44 SE and 43 ± 7 SE, respectively) and gender (both groups included 4 males and 2 females).

Characterization of (neuron-derived) exosomes

Plasma exosomes were analyzed for size, morphology and exosomal markers using NTA, TEM and Western blotting, respectively (Figure 1). NDEs were only used for NTA since their yield was too low for TEM or Western blotting. NTA revealed that the size range of total exosomes (bulk ~108-300nm) was wider than that of NDEs (bulk 100-200nm; Figure

1 A.1 and A.2). ExoQuick isolated 1.20 x 10^9 particles/ml total exosomes and 8.76 x 10^8 particles/ml NDEs. TEM yielded images that were clouded by co-precipitants, suggesting the presence of other proteins, vesicles or ExoQuick-related debris (Figure 1B). Western blotting confirmed the presence of the exosome markers CD63, TSG101 and Alix, along with the absence of the cellular marker calnexin (Figure 1C).

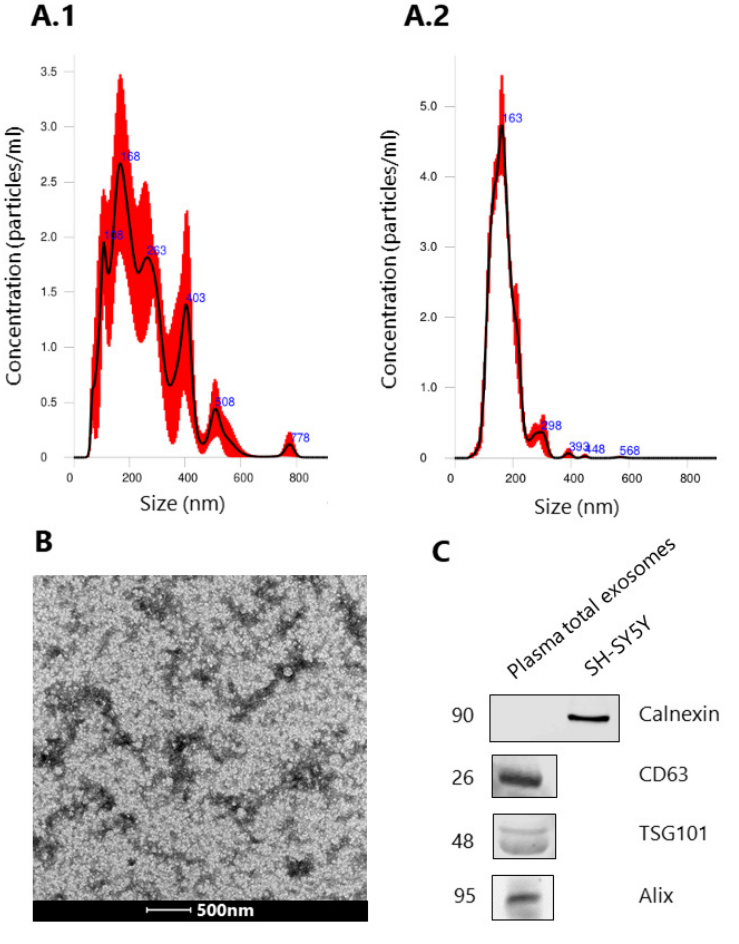


Figure 1. Plasma (neuron-derived) exosome characterization and size determination. (A) Nanoparticle Tracking Analysis showing the size range of total exosomes (A.1) and NDEs (A.2). Note that different sample dilutions were used for total exosomes and NDEs. (B) Transmission electron microscopy (TEM) showing the presence of co-precipitants when using ExoQuick on plasma. Scale 500nm. (C) Western blotting showing the presence of exosomal markers (CD63, TSG101 and Alix) and the absence of calnexin in total exosome preparations, along with the presence of calnexin in SH-SY5Y cell lysates.

Neuron-derived exosome miRNA sequencing and differential expression analysis

A total average of 4.6 million raw reads were obtained per sample, the majority of which remained unmapped (~67%; Table 1). Most of the mapped reads aligned to ncRNAs other than miRNAs (55.7%), followed by miRNAs (23%) and ribosomal RNA (rRNA; 19.7%). Reads that mapped to miRNAs revealed the presence of 561 different mature miRNAs (average of 297 ± 39.5 SD per sample). For further analyses, we only retained those miRNAs with an average of at least 10 counts across samples, which resulted in 265 distinct miRNAs (Table 1).

Two miRNAs, miR-581-5p and miR-542-3p, were significantly differentially expressed between PTSD subjects and controls (*p*-adj=0.04 and 0.06, respectively; Figure 2A). Both miRNAs were upregulated in NDEs from cases as compared to controls (log2 fold changes (FC) of 5.3 and 6.2, respectively). Both miRNAs were consistently expressed in all 6 PTSD samples, while remaining undetected in 5 out of the 6 control samples.

Receiver operating characteristic (ROC) curve analysis revealed that the area under the curves (AUCs) for these miRNAs were 0.97 for miR-581-5p and 0.90 for miR-542-3p (Figure 2 B.1 and B.2). While miR-581-5p distinguished cases from controls with 100% sensitivity and 83% specificity, miR-542-3p did so with 83% sensitivity and 100% specificity.

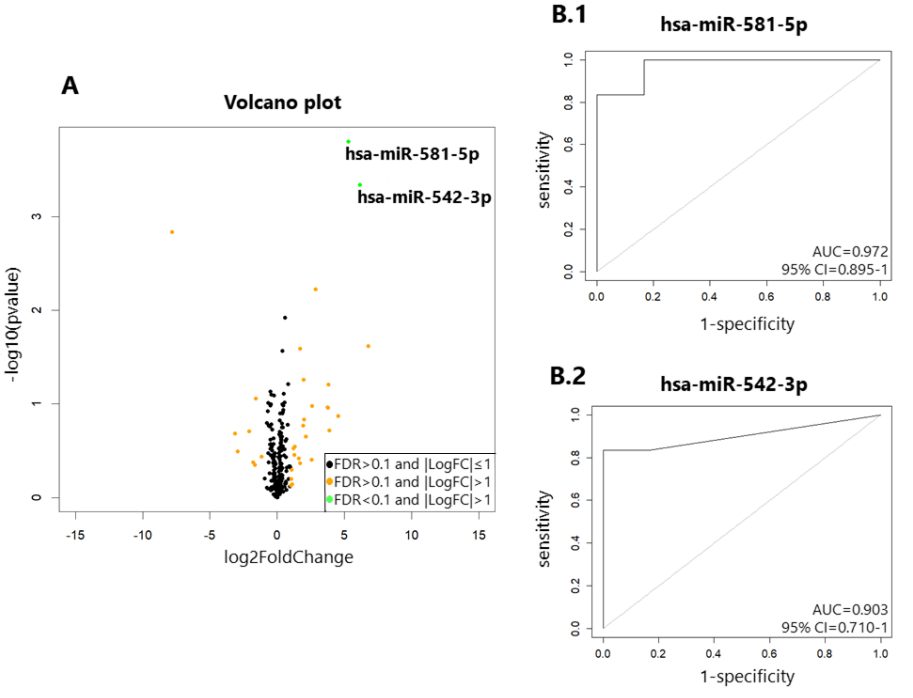


Figure 2. Identification of differentially expressed miRNAs from plasma NDEs. (A) Volcano plot showing all expressed miRNAs. Black dots represent miRNAs with a log2 FC ≤ |1|, of which the expression was not significantly different between both groups. Orange dots represent miRNAs with a log2 FC > |1|, of which the expression is not significantly different between both groups. Green dots represent differentially expressed miRNAs with a log2 FC > |1|. Significance is declared when adjusted *p*-value <0.1. (B) Receiver operating characteristic (ROC) curves for miR-581-5p (B.1) and miR-542-3p (B.2) for the diagnosis of PTSD with corresponding Areas Under the Curve (AUC) and 95% Confidence Intervals (CI).

Target gene pathway and gene ontology analyses

Together, both miRNAs targeted 231 genes (miR-581 targets 57 genes, miR-542-3p targets 176 genes, 2 genes are in common). We identified 13 enriched pathways, including the HIF-1 signaling pathway, next to several cancer- and immune-related pathways (Supplementary Table S2). GO analyses for Biological Processes identified the most enrichment for "regulation of lipid metabolic process", "cellular response to hypoxia, "DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest" and "protein ubiquitination" (Supplementary Table S3). GO analyses of Molecular Functions identified the following top enrichments: "Poly(A) RNA binding", "Nucleosome binding", "Ubiquitin-protein transferase activity", and "Enzyme binding" (Supplementary Table S4). Finally, GO analyses of the Cellular Component category identified "Nucleus", "Cytoplasm", "Nucleoplasm", "PCNA-p21 complex" and "Cytosol" (Supplementary Table S5).

CESAMH: Urine neuron-derived exosomes

We used a slightly adapted exosome isolation protocol to assess whether NDEs could also be isolated from urine samples belonging to the same individuals. The recovered particles were of the expected size and morphology for exosomes (Figure 3A and B).

Neuron-derived exosome miRNA sequencing and differential expression analysis

Out of a total average of 4.9 million raw reads per sample, most remained unmapped (86%; Table 1). Mapped reads aligned to rRNA (54.5%), miRNAs (22.6%), transfer RNAs (tRNAs; 12.3%) and other non-coding RNAs (9.6%). A total of 190 different mature miRNAs were detected (average of 75.3 ± 20.6 SD per sample) of which 81 had an average read count of 10 or more across samples (Table 1).

None of these miRNAs were differentially expressed between cases and control individuals. Comparing the 50 most abundant miRNAs in plasma and urine revealed that 28 miRNAs (56%) were present in both datasets. When combining urine and plasma miRNAs expression profiles, Principal Component Analysis (PCA) showed that the different types of biofluids explain 69% of variance (Figure 3C). When correlating expression profiles from miRNAs detected in both plasma and in urine, regardless of average count number across samples (N=177), only 12 were significantly correlated with correlation coefficients ranging between |0.61| and |0.88| (11 were positively correlated, one negatively; Supplementary Table S6).

Table 1. Overview of input volume, (microRNA) reads and counts per biofluid.

	Input vol (mL)	**Average # of million raw reads per sample (SD)**	**Average % of un-mapped reads (SD)**	**Average % of reads mapping to miRNAs (SD)**	**Average # of mature miRNAs per sample (SD)**	**Total # of mature miRNAs**	**Total # of mature miRNAs (> 10 counts)**
Plasma	0.75	4.6 (0.9)	67.5 (10.7)	23.1 (4.1)	297 (39.5)	561	265
Urine	1.6	4.9 (0.8)	86.4 (3.7)	22.6 (6)	75.3 (20.6)	190	81
Serum	0.5	3.2 (1.4)	78.6 (2.6)	37.8 (3.9)	112 (33.1)	345	158

Note: direct comparisons between biofluids should be made carefully considering differences such as input volumes and library prep kits. Vol: Volume, SD: standard deviation.

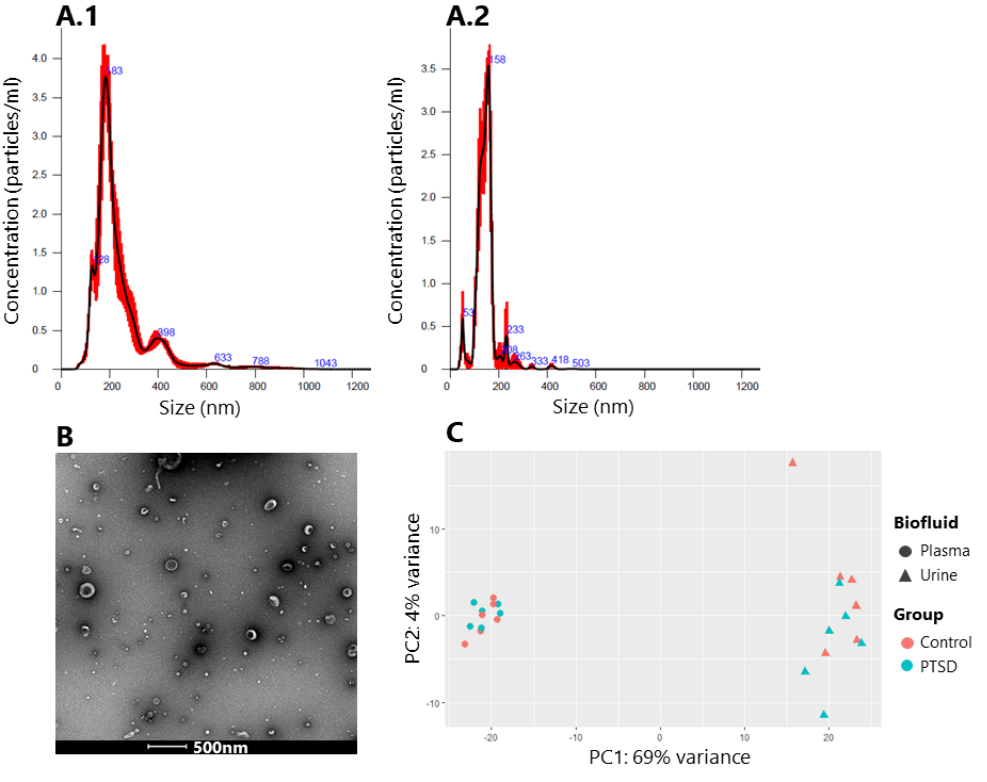


Figure 3. Urine (neuron-derived) exosome characterization, size determination and comparison to plasma. (A) Nanoparticle Tracking Analysis showing the size range of total exosomes (A.1) and NDEs (A.2). (B) Transmission electron microscopy (TEM) showing the presence of urine exosomes. Scale 500nm. (C) Color-labeled PCA plot of urine (triangles) and plasma (rounds) miRNA expression profiles (red: controls, blue: PTSD cases).

PRISMO: Serum neuron-derived exosomes

Demographics

Three groups belonging to the PRISMO cohort were included; individuals with PTSD, trauma-exposed individuals without PTSD (i.e. "resilient" individuals) and non-exposed control individuals (N=24, 8 per group). The three groups were matched for age and did not differ in terms of number of previous deployments (Supplementary Table S1). On average, subjects with PTSD and resilient individuals were exposed to a similar amount of traumatic events, which was significantly higher than the non-exposed controls ($\chi^2(2) = 15.78$, $p<.001$. Dunn's post-hoc showed $p=.002$ for PTSD vs control, and resilient vs control). Resilient and control subjects had similar post-deployment PTSD scores as measured by the SRIP which were significantly lower than the average score of the PTSD group ($\chi^2(2) = 15.5$, $p<.001$. Dunn's post-hoc showed $p=.002$ for PTSD vs resilient, and $p=.003$ for PTSD vs control; Supplementary Table S1).

Neuron-derived exosome miRNA sequencing and differential expression analysis

A total average of 3.2 million raw reads were obtained per sample, the majority of which remained unmapped (~78%; Table 1). Out of the mapped reads, most reads mapped to rRNA (41.7%), followed by miRNAs (37.8%), other non-coding RNAs (17.5%) and tRNAs (1.3%). Reads that mapped to miRNAs revealed the presence of 345 different mature miRNAs (average of 112 ± 33.1 SD per sample). Retaining only those miRNAs with an average of at least 10 counts across samples resulted in 158 distinct miRNAs (Table 1).

None of these miRNAs were differentially expressed between individuals with PTSD and trauma-exposed controls or healthy controls. Comparing the 50 most abundant miRNAs in plasma and serum revealed that 36 miRNAs (72%) were present in both datasets. Strikingly, read length distributions showed a great amount of small RNA fragments, as opposed to an expected and distinct peak around 22nt corresponding to miRNAs as seen in our plasma samples (Figure 4).

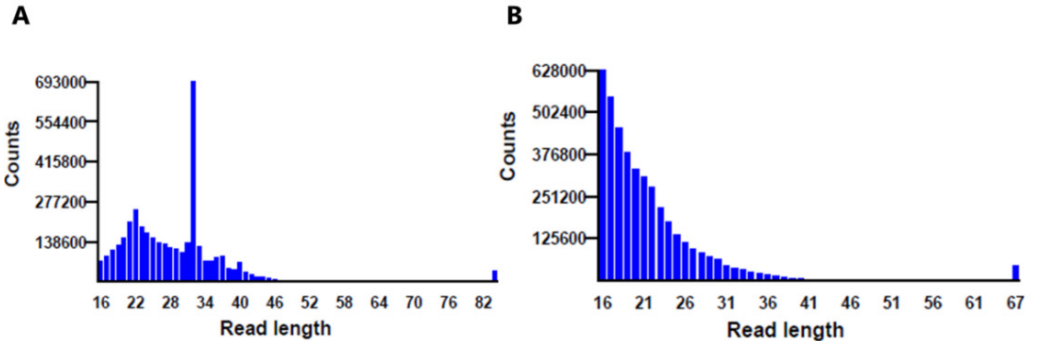


Figure 4. Read length distributions of a plasma (A) and serum (B) sample.

Discussion

PTSD is a debilitating psychiatric disorder which lacks appropriate treatment options. Diving into the (neuro)biological underpinnings of this disorder is much needed as it will allow us to gain insights into key molecular players which could potentially prove to serve as effective biomarkers, and yield relevant information regarding potential new treatment strategies. In this pilot study, we aimed to obtain proof-of-principle for characterizing the miRNA content of plasma NDEs and test for differential expression of NDE-derived miRNAs in PTSD. Additionally, we aimed to (i) assess whether urine carries NDEs and a similar protocol could be used to explore their miRNA contents, and (ii) assess whether serum samples, which were stored for about 10 to 15 years, could also serve as starting material for NDE miRNA isolation and possibly replicate our plasma findings.

First, we characterized plasma exosomes using a combination of NTA, Western blotting and TEM. NDEs were only used for NTA since their yield was too low for TEM or Western blotting. Assessing particle sizes by NTA revealed that most particles isolated by ExoQuick were between 108 and ~300nm in size, which includes the expected size range for (neuron-derived) exosomes and is comparable to previous findings [41, 42]. However, the larger particles suggest the potential presence of larger non-exosomal extracellular vesicles or exosome aggregates. Of interest here is that isolating NDEs greatly reduced the size heterogeneity, pointing to a relative size specificity of L1CAM+ exosomes. Furthermore, the exosome markers CD63, TSG101 and Alix confirmed the presence of exosomes, while the absence of calnexin, a marker for the endoplasmic reticulum, points to the absence of cellular contaminants. TEM preparations of total exosomes revealed blurred images similar to previously reported findings using ExoQuick on plasma samples [41, 42]. These findings strengthen the previously postulated notion that using ExoQuick

on plasma might not be suitable for TEM due to the presence of co-precipitants, which are potentially related to the reagent itself [41].

Differential expression analyses revealed that miR-581-5p and miR-542-3p were upregulated in PTSD subjects as compared to controls and had a high diagnostic potential for PTSD as reflected by high AUCs, and the high sensitivity and specificity with which they differentiate PTSD cases from control individuals. Although small sample sizes are known to be associated with less specific ROC curves [43], replication in larger samples is warranted, and caution is needed when interpreting these findings, it remains striking that both miRNAs were detected across all PTSD samples while remaining absent in five out of six control samples.

While the precise roles of both miRNAs are not well understood yet, our pathway and GO analyses using their 231 combined predicted gene targets, showed enrichment of genes in pathways and several biological processes related to hypoxia and cell damage. The transcription factor Hypoxia-inducible factor 1 (HIF-1) is known to regulate angiogenesis and the cellular response to hypoxia, and is critical during embryonic development, specifically during the neural fold formation [44, 45]. Ischemia-hypoxia during obstetric complications has been shown to interfere with proper neuronal development and to increase the risk of schizophrenia development later in life [46, 47]. Studies have also identified significant overlap between gene sets related to ischemia-hypoxia and schizophrenia [48], and reported associations between increased mRNA expression of HIF-1, major depressive disorder and bipolar disorder [49]. Through their targeted genes, miR-581 and miR-542-3p are thus suggested to be involved in hypoxia-related pathways, which could contribute to psychiatric outcomes such as PTSD. Other studies found miR-581 involvement in bipolar disorder [50] and liver cancer [51], while miR-542-3p has more recently been found downregulated in the medial prefrontal cortex of FKBP5 knockdown mice [52], upregulated in cultured dermal fibroblasts of patients with major depressive disorder [53], involved Parkinson's disease [54] and in cancer-related processes [55, 56]. No other study found implications of these miRNAs in PTSD.

We further assessed whether, next to plasma, urine also contains NDEs. Urine is easily accessible and could thus be collected and analyzed more easily in prospective cohort studies. We were able to obtain L1CAM+ exosomes and to extract and sequence their miRNA content. No miRNAs were differentially expressed between PTSD cases and controls, and correlation coefficients of NDE miRNA content between urine and plasma

remained very low and insignificant for most of the commonly detected miRNAs. However, since it was recently shown that L1CAM is also expressed in the epithelia of the kidney [57], it is possible that the origin of urine L1CAM+ exosomes is not limited to the brain, which could help explain the limited overlap in miRNAs between biofluids despite originating from the same individual. Although online databases show that L1CAM is highly and mostly expressed by neurons (https://www.proteinatlas.org/ENSG00000198910-L1CAM/tissue), we also cannot entirely rule out that a fraction of our plasma and serum L1CAM+ exosomes could originate from other organs. To further asses the feasibility of using urine as a reliable source of NDEs, future work should investigate the specificity of L1CAM for the isolation of NDEs from urine exosome samples.

The serum NDEs we studied next, contained an unexpectedly large number of fragments smaller than 18 nucleotides in length. This may point to sample degradation, i.e. the breakdown of longer RNA species such as rRNA and messenger RNA (mRNA). Based on previous reports showing high stability of miRNAs, even despite (near) total mRNA degradation [58, 59], it is safe to assume that the miRNA content of our serum samples is still mostly preserved. This is further supported by the fact that we found 72% of overlap in abundant miRNAs between our plasma and serum datasets (when considering the 50 most expressed miRNAs within each dataset), which also greatly overlapped with previously published datasets of miRNA abundance in blood exosomes [60, 61]. For example, out of the 20 most abundant plasma exosome miRNAs reported by Aae et al. [60], 15 were among the most abundant in both our plasma and serum datasets, 4 were present in one of the datasets, and only one was not present in either dataset. Since these published studies examined total exosome populations, these results do suggest that blood exosomes carry roughly the same abundant miRNAs, regardless of their tissue of origin.

Some aspects regarding the sequencing output need to be highlighted. First, the relatively small fraction of reads mapping to miRNAs (~23-38%) is comparable to previous findings reporting blood or urine exosomal small RNA sequencing data [61-64]. Similar to our findings, one of these studies also reported a larger number of miRNA reads within serum exosomes as compared to plasma exosomes [61]. However, regardless of the biofluid, the large majority of our reads remained unmapped. Although relatively large amounts of unmapped reads within blood and urine exosomes were reported before (e.g. ~45% and 20-33% in plasma [63, 65], 49% and 40% in urine [66, 67]), the numbers of unmapped reads presented here are substantially larger. Since the

only other study examining human blood NDE miRNA profiles did so using quantitative PCR of a few select miRNAs [68] as opposed to NGS, there currently is not enough data available to compare our findings to similar datasets. This further means that no *a priori* knowledge on NDE miRNA content is available to compare to the amount of mapped reads or distinct miRNAs we detect within our blood, let alone urine, NDE samples. Together, our sequencing findings show that (i) old serum samples likely contain more degraded RNA fragments, although degradation of miRNAs, specifically, might be less likely, (ii) our plasma and serum NDEs share most of their abundant miRNAs, which, in turn, overlap with published datasets on blood total exosome miRNA findings, and (iii) more NDE-specific miRNA studies are needed in order to directly compare the present findings and unexpected aspects such as the large amount of unmapped reads.

In conclusion, this pilot study is the first to suggest that (i) miRNAs encapsulated within plasma NDEs can be isolated and analyzed by sequencing using limited amounts of starting material, and could potentially, if replicated in larger cohorts, serve as markers for combat-related PTSD, (ii) more research is needed in order to assess the utility of urine samples for these types of analyses, and (iii) older serum samples might still be useful for NDE miRNA analyses, although future studies are needed to study miRNA stability despite evidence pointing to sample degradation. Given the limited sample sizes used in the present study, larger follow-up studies are now needed in order to replicate and build upon these preliminary findings and further assess the functional roles of any differentially expressed miRNA.

References

1. American Psychiatric Association. and American Psychiatric Association. DSM-5 Task Force., *Diagnostic and statistical manual of mental disorders : DSM-5*. 5th ed. 2013, Washington, D.C.: American Psychiatric Association. xliv, 947 p.
2. Danielsson, F.B., et al., *Quality of life and level of post-traumatic stress disorder among trauma patients: A comparative study between a regional and a university hospital.* Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine, 2018. **26**(1): p. 44.
3. Yehuda, R., et al., *The use of biomarkers in the military: from theory to practice.* Psychoneuroendocrinology, 2013. **38**(9): p. 1912-22.
4. Lehrner, A. and R. Yehuda, *Biomarkers of PTSD: military applications and considerations.* Eur J Psychotraumatol, 2014. **5**.
5. Hoge, C.W., et al., *PTSD treatment for soldiers after combat deployment: low utilization of mental health care and reasons for dropout.* Psychiatr Serv, 2014. **65**(8): p. 997-1004.
6. Kloosterman, W.P. and R.H. Plasterk, *The diverse functions of microRNAs in animal development and disease.* Dev Cell, 2006. **11**(4): p. 441-50.
7. Rajasethupathy, P., et al., *Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB.* Neuron, 2009. **63**(6): p. 803-17.
8. Hou, Q., et al., *MicroRNA miR124 is required for the expression of homeostatic synaptic plasticity.* Nat Commun, 2015. **6**: p. 10045.
9. Schratt, G.M., et al., *A brain-specific microRNA regulates dendritic spine development.* Nature, 2006. **439**(7074): p. 283-9.
10. Fiorenza, A., et al., *Blocking miRNA Biogenesis in Adult Forebrain Neurons Enhances Seizure Susceptibility, Fear Memory, and Food Intake by Increasing Neuronal Responsiveness.* Cereb Cortex, 2016. **26**(4): p. 1619-1633.
11. Konopka, W., et al., *MicroRNA loss enhances learning and memory in mice.* J Neurosci, 2010. **30**(44): p. 14835-42.
12. Sillivan, S.E., et al., *MicroRNA regulation of persistent stress-enhanced memory.* Mol Psychiatry, 2020. **25**(5): p. 965-976.
13. Arroyo, J.D., et al., *Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma.* Proc Natl Acad Sci U S A, 2011. **108**(12): p. 5003-8.
14. Michell, D.L. and K.C. Vickers, *Lipoprotein carriers of microRNAs.* Biochim Biophys Acta, 2016. **1861**(12 Pt B): p. 2069-2074.
15. Snijders, C., et al., *MicroRNAs in Post-traumatic Stress Disorder.* Curr Top Behav Neurosci, 2018. **38**: p. 23-46.
16. Van den Brande, S., et al., *The presence of extracellular microRNAs in the media of cultured Drosophila cells.* Scientific Reports, 2018. **8**(1): p. 17312.
17. Turchinovich, A., et al., *Characterization of extracellular circulating microRNA.* Nucleic Acids Res, 2011. **39**(16): p. 7223-33.
18. Caby, M.P., et al., *Exosomal-like vesicles are present in human blood plasma.* Int Immunol, 2005. **17**(7): p. 879-87.
19. Yagi, Y., et al., *Next-generation sequencing-based small RNA profiling*

of cerebrospinal fluid exosomes. Neurosci Lett, 2017. **636**: p. 48-57.
20. Conde-Vancells, J. and J.M. Falcon-Perez, *Isolation of urinary exosomes from animal models to unravel noninvasive disease biomarkers.* Methods Mol Biol, 2012. **909**: p. 321-40.
21. Théry, C., L. Zitvogel, and S. Amigorena, *Exosomes: composition, biogenesis and function.* Nature Reviews Immunology, 2002. **2**(8): p. 569-579.
22. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nature Cell Biology, 2007. **9**(6): p. 654-659.
23. Jaiswal, R. and L.M. Sedger, *Intercellular Vesicular Transfer by Exosomes, Microparticles and Oncosomes - Implications for Cancer Biology and Treatments.* Frontiers in oncology, 2019. **9**: p. 125-125.
24. Wu, D., et al., *Profiling surface proteins on individual exosomes using a proximity barcoding assay.* Nat Commun, 2019. **10**(1): p. 3854.
25. Winston, C.N., et al., *Prediction of conversion from mild cognitive impairment to dementia with neuronally derived blood exosome protein profile.* Alzheimers Dement (Amst), 2016. **3**: p. 63-72.
26. Kapogiannis, D., et al., *Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease.* FASEB J, 2015. **29**(2): p. 589-96.
27. Goetzl, E.J., et al., *Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease.* FASEB J, 2016. **30**(12): p. 4141-4148.
28. Fiandaca, M.S., et al., *Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study.* Alzheimers Dement, 2015. **11**(6): p. 600-7 e1.
29. Sun, B., et al., *Blood neuron-derived exosomes as biomarkers of cognitive impairment in HIV.* AIDS (London, England), 2017. **31**(14): p. F9-F17.
30. van der Wal, S.J., et al., *Cohort profile: the Prospective Research In Stress-Related Military Operations (PRISMO) study in the Dutch Armed Forces.* BMJ Open, 2019. **9**(3): p. e026670.
31. Reijnen, A., et al., *Prevalence of mental health symptoms in Dutch military personnel returning from deployment to Afghanistan: a 2-year longitudinal analysis.* Eur Psychiatry, 2015. **30**(2): p. 341-6.
32. Hovens, J.E., et al., *The development of the Self-Rating Inventory for Posttraumatic Stress Disorder.* Acta Psychiatr Scand, 1994. **90**(3): p. 172-83.
33. Laurent, L.C. and R.P. Alexander, *RNA isolation using the miRNeasy Micro Kit.* 2015.
34. Baras, A.S., et al., *miRge - A Multiplexed Method of Processing Small RNA-Seq Data to Determine MicroRNA Entropy.* PLOS ONE, 2015. **10**(11): p. e0143066.
35. Kozomara, A. and S. Griffiths-Jones, *miRBase: annotating high confidence microRNAs using deep sequencing data.* Nucleic Acids Res, 2014. **42**(Database issue): p. D68-73.
36. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
37. Benjamini, Y., Hochberg, Y., *Controlling The False Discovery Rate: A Practical And Powerful Approach To Multiple Testing.* Journal of the Royal Statistical Society, 1995. **57**: p. 289-300.

38. Chou, C.H., et al., *miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions*. Nucleic Acids Res, 2018. **46**(D1): p. D296-D302.
39. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat Protoc, 2009. **4**(1): p. 44-57.
40. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists*. Nucleic Acids Res, 2009. **37**(1): p. 1-13.
41. Serrano-Pertierra, E., et al., *Characterization of Plasma-Derived Extracellular Vesicles Isolated by Different Methods: A Comparison Study*. Bioengineering (Basel), 2019. **6**(1).
42. Soares Martins, T., et al., *Exosome isolation from distinct biofluids using precipitation and column-based approaches*. PloS one, 2018. **13**(6): p. e0198820-e0198820.
43. Min, L., et al., *Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs*. J Extracell Vesicles, 2019. **8**(1): p. 1643670.
44. Cejudo-Martin, P. and R.S. Johnson, *A new notch in the HIF belt: how hypoxia impacts differentiation*. Dev Cell, 2005. **9**(5): p. 575-6.
45. Scully, D., et al., *Hypoxia promotes production of neural crest cells in the embryonic head*. Development, 2016. **143**(10): p. 1742-52.
46. Mittal, V.A., L.M. Ellman, and T.D. Cannon, *Gene-environment interaction and covariation in schizophrenia: the role of obstetric complications*. Schizophr Bull, 2008. **34**(6): p. 1083-94.
47. Van Erp, T.G., et al., *Contributions of genetic risk and fetal hypoxia to hippocampal volume in patients with schizophrenia or schizoaffective disorder, their unaffected siblings, and healthy unrelated volunteers*. Am J Psychiatry, 2002. **159**(9): p. 1514-20.
48. Schmidt-Kastner, R., et al., *Analysis of GWAS-Derived Schizophrenia Genes for Links to Ischemia-Hypoxia Response of the Brain*. Front Psychiatry, 2020. **11**: p. 393.
49. Shibata, T., et al., *The alteration of hypoxia inducible factor-1 (HIF-1) and its target genes in mood disorder patients*. Prog Neuropsychopharmacol Biol Psychiatry, 2013. **43**: p. 222-9.
50. Forstner, A.J., et al., *Genome-wide analysis implicates microRNAs and their target genes in the development of bipolar disorder*. Transl Psychiatry, 2015. **5**: p. e678.
51. Katayama, Y., et al., *Identification of pathogenesis-related microRNAs in hepatocellular carcinoma by expression profiling*. Oncol Lett, 2012. **4**(4): p. 817-823.
52. Kang, H.J., et al., *FKBP5-associated miRNA signature as a putative biomarker for PTSD in recently traumatized individuals*. Scientific Reports, 2020. **10**(1): p. 3353.
53. Garbett, K.A., et al., *Coordinated Messenger RNA/MicroRNA Changes in Fibroblasts of Patients with Major Depression*. Biological Psychiatry, 2015. **77**(3): p. 256-265.
54. Mo, M., et al., *MicroRNA expressing profiles in A53T mutant alpha-synuclein transgenic mice and Parkinsonian*. Oncotarget, 2017. **8**(1): p. 15-28.
55. Wang, X.-P., et al., *MicroRNA-542-3p functions as a tumor suppressor via directly targeting survivin in hepatocellular carcinoma*.

Biomedicine & Pharmacotherapy, 2018. **99**: p. 817-824.
56. Wang, W.-T., et al., *Differentially expressed microRNAs in the serum of cervical squamous cell carcinoma patients before and after surgery.* Journal of Hematology & Oncology, 2014. **7**(1): p. 6.
57. Pechriggl, E.J., et al., *L1CAM in the Early Enteric and Urogenital System.* J Histochem Cytochem, 2017. **65**(1): p. 21-32.
58. Hall, J.S., et al., *Enhanced stability of microRNA expression facilitates classification of FFPE tumour samples exhibiting near total mRNA degradation.* Br J Cancer, 2012. **107**(4): p. 684-94.
59. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
60. Aae, T.F., et al., *Evaluating plasma extracellular vesicle microRNAs as possible biomarkers for osteoarthritis.* Osteoarthritis and Cartilage Open, 2020. **1**(3): p. 100018.
61. Cheng, L., et al., *Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood.* J Extracell Vesicles, 2014. **3**.
62. Srinivasan, S., et al., *Assessment of methods for serum extracellular vesicle small RNA sequencing to support biomarker development.* J Extracell Vesicles, 2019. **8**(1): p. 1684425.
63. Sundar, I.K., D. Li, and I. Rahman, *Small RNA-sequence analysis of plasma-derived extracellular vesicle miRNAs in smokers and patients with chronic obstructive pulmonary disease as circulating biomarkers.* J Extracell Vesicles, 2019. **8**(1): p. 1684816.
64. Cheng, L., et al., *Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine.* Kidney International, 2014. **86**(2): p. 433-444.
65. Huang, X., et al., *Characterization of human plasma-derived exosomal RNAs by deep sequencing.* BMC Genomics, 2013. **14**: p. 319.
66. Gracia, T., et al., *Urinary Exosomes Contain MicroRNAs Capable of Paracrine Modulation of Tubular Transporters in Kidney.* Scientific Reports, 2017. **7**(1): p. 40601.
67. Rodríguez, M., et al., *Identification of non-invasive miRNAs biomarkers for prostate cancer by deep sequencing analysis of urinary exosomes.* Molecular Cancer, 2017. **16**(1): p. 156.
68. Cha, D.J., et al., *miR-212 and miR-132 Are Downregulated in Neurally Derived Plasma Exosomes of Alzheimer's Patients.* Front Neurosci, 2019. **13**: p. 1208.

Supplementary material

Supplementary Table S1. Demographic characteristics of the PRISMO cohort.

	PTSD (N=8)	**Resilient (N=8)**	**Control (N=8)**	***P*-value**
Age when deployed	26.1 (11.4)	32.8 (11.4)	23.4 (2.6)	.254
Trauma exposure-score	7.75 (2.8)	7.75 (1.9)	0.5 (0.5)	<.001
SRIP PTSD score	53.1 (12.7)	25.4 (3.7)	25.3 (2.9)	<.001
Number of previous deployments	0.86 (1.5)	1.13 (1.6)	0.13 (0.4)	.229

Data is presented as mean (standard deviation (SD)). SRIP: Self-Rating Inventory for Post-Traumatic Stress Disorder.

Supplementary Table S2. KEGG pathways enriched for the 231 target genes of miR-581 and miR-542-3p.

Term	***P*-value** ***Genes***	**FDR adj *p*-value**
Proteoglycans in cancer	0.004	0.297
	CDKN1A, CTTN, FZD7, MDM2, PLCG2, AKT1, RAC1, HPSE, PIK3R1	
HIF-1 signaling pathway	0.008	0.297
	ANGPT4, CDKN1A, ANGPT2, PLCG2, AKT1, PIK3R1	
Central carbon metabolism in cancer	0.009	0.297
	SLC7A5, PGAM1, PGAM4, AKT1, PIK3R1	
Glioma	0.009	0.297
	CDKN1A, MDM2, PLCG2, AKT1, PIK3R1	
Hepatitis B	0.010	0.297
	MAP2K4, CDKN1A, PCNA, YWHAB, BIRC5, AKT1, PIK3R1	
Fc epsilon RI signaling pathway	0.011	0.297
	MAP2K4, PLCG2, AKT1, RAC1, PIK3R1	
Hippo signaling pathway	0.013	0.297
	PARD6B, YWHAB, FZD7, ID2, BIRC5, NF2, BMP7	
Epstein-Barr virus infection	0.020	0.408
	MAP2K4, CDKN1A, MDM2, PLCG2, AKT1, PIK3R1	
ErbB signaling pathway	0.025	0.459
	MAP2K4, CDKN1A, PLCG2, AKT1, PIK3R1	
Acute myeloid leukemia	0.034	0.534
	PIM1, AKT1, PIK3R1, RUNX1T1	
VEGF signaling pathway	0.043	0.534
	PLCG2, AKT1, RAC1, PIK3R1	
Colorectal cancer	0.045	0.534
	BIRC5, AKT1, RAC1, PIK3R1	
Toll-like receptor signaling pathway	0.046	0.534
	MAP2K4, AKT1, RAC1, PIK3R1, TLR5	

Pathways were identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S3. Gene ontology (GO) biological process analysis of the 231 target genes of miR-581 and miR-542-3p.

GO term **GO ID**	***P*-value** ***Genes***	**FDR adj** ***p*-value**
Regulation of lipid metabolic process GO:0019216	0.004 *ID2, OPA3, IRS2, TM6SF2*	1
Cellular response to hypoxia GO:0071456	0.007 *ANGPT4, MDM2, AKT1, MDM4, BMP7, ZFP36L1*	1
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest GO:0006977	0.007 *CDKN1A, PCNA, MDM2, MDM4, AURKA*	1
Protein ubiquitination GO:0016567	0.014 *MYLIP, FEM1A, TRIM28, KLHL15, MDM2, IPP, KLHL21, BIRC5, AKT1, MDM4, RNF4*	1
Cell proliferation GO:0008283	0.016 *PURB, PCNA, INSIG1, PIM1, ILK, AKT1, MDM4, IRS2, TACC2, RAC1, ZFP36L1*	1
Insulin receptor signaling pathway GO:0008286	0.016 *IGFBP1, AKT1, IRS2, PIK3R1, ATP6V1E1*	1
Centrosome localization GO:0051642	0.016 *RANBP2, SUN2, AURKA*	1
Protein autophosphorylation GO:0046777	0.020 *NTRK2, TRIM28, PIM1, AKT1, PTK6, EPHB4, AURKA*	1
Neuron projection morphogenesis GO:0048812	0.022 *CTTN, ILK, DICER1, BMP7*	1
Negative regulation of cell cycle arrest GO:0071157	0.025 *MDM2, MDM4, ZNF268*	1
Negative regulation of apoptotic process GO:0043066	0.027 *PLAC8, ANGPT4, CDKN1A, MDM2, PIM1, BIRC5, AKT1, MDM4, PIK3R1, ZNF268, AURKA, MTDH*	1
Positive regulation of cell migration GO:0030335	0.027 *SPAG9, SUN2, SEMA3G, ILK, IRS2, PIK3R1, ZNF268*	1
RNA processing GO:0006396	0.032 *TRUB2, ATXN1, HNRNPUL1, TRMT2B, DICER1*	1
Cellular senescence GO:0090398	0.033 *CDKN1A, ID2, TERF2*	1
Cellular response to hydrogen peroxide GO:0070301	0.034 *KDM6B, IMPACT, PCNA, MDM2*	1
Cellular response to UV-C GO:0071494	0.037 *IMPACT, MDM2*	1
Translation GO:0006412	0.038 *MRPS17, RPS28, WARS, RPL36A-HNRNPH2, AKT1, SLC25A43, RPL28, RPS23*	1
Response to wounding GO:0009611	0.043 *MAP2K4, RAC1, ZFP36L1, AURKA*	1
Metanephros development GO:0001656	0.044 *RDH10, ID2, BMP7*	1
	0.045	1

Positive regulation of proteasomal ubiquitin-dependent protein catabolic process GO:0032436	*MDM2, AKT1, PRICKLE1, AURKA*	
Positive regulation of mitotic cell cycle GO:0045931	0.047	1
	POLDIP2, MDM2, BIRC5	
Protein import into nucleus, translocation GO:0000060	0.047	1
	AKT1, KPNB1, RBM22	
Negative regulation of plasma membrane long-chain fatty acid transport GO:0010748	0.049	1
	AKT1, IRS2	

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S4. Gene ontology (GO) molecular function analysis of the 231 target genes of miR-581 and miR-542-3p.

GO term **GO ID**	***P*-value** ***Genes***	**FDR adj *p*-value**
Poly(A) RNA binding GO:0044822	0.003	0.960
	TRUB2, RTCB, ZFP36L1, MTDH, PURB, TRIM28, KIAA1324, G3BP2, SMNDC1, PURG, SUPT16H, HNRNPA3, UTP4, CDC5L, LARP4B, RPS28, GNL3L, HNRNPUL1, ESRP2, CSDE1, ALDOA, PPIL4, RPL28, KPNB1, RBM22, RPS23	
Nucleosome binding GO:0031491	0.016	1
	SUPT16H, SMARCA5, RNF4	
Ubiquitin-protein transferase activity GO:0004842	0.019	1
	MYLIP, FEM1A, TRIM28, KLHL15, MGRN1, MDM2, IPP, KLHL21, BIRC5, RNF4	
Enzyme binding GO:0019899	0.021	1
	BTG1, PCNA, HNRNPUL1, YWHAB, MDM2, BIRC5, AKT1, MDM4, RAC1, KPNB1	
Protein C-terminus binding GO:0008022	0.024	1
	HIC2, ATXN1, YWHAB, CACNA1B, SYNJ2BP, TERF2, FBLN5	
14-3-3 protein binding GO:0071889	0.034	1
	AKT1, IRS2, ZFP36L1	
Protein phosphatase binding GO:0019903	0.041	1
	PPP6R3, SHOC2, IRS2, PIK3R1	
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase activity GO:0046538	0.048	1
	PGAM1, PGAM4	
Phosphoglycerate mutase activity GO:0004619	0.048	1
	PGAM1, PGAM4	
Bisphosphoglycerate mutase activity GO:0004082	0.048	1
	PGAM1, PGAM4	
Protein serine/threonine/tyrosine kinase activity GO:0004712	0.048	1
	DYRK3, AKT1, AURKA	

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S5. Gene ontology (GO) cellular component analysis of the 231 target genes of miR-581 and miR-542-3p.

GO term	***P*-value**	**FDR adj**
GO ID	***Genes***	***p*-value**
Nucleus	2.03E-06	5.61E-04
GO:0005634	*GABPB2, RTCB, PRDM2, CDC14B, PNP, SESN3, TRIM28, PIM1, AKT1, PPP1R16B, MAP2K4, ZNF682, ZNF285, SUPT16H, DICER1, HIC2, MT1A, HNRNPUL1, HAT1, ZNF555, ZNF157, RNASEH2B, SPATA2, PIK3R1, ZBTB3, ZFP36L1, MTDH, ATXN1, RDH10, ZNF268, TRAPPC2, SMARCA5, DCDC2, PTK6, GNL3L, EHD4, EIF2S3, ID2, ZNF417, MDM2, MDM4, HPSE, NF2, BRWD1, CDKN1A, DYRK3, BTG1, YWHAB, FAM208B, CDC73, NRGN, CHAF1B, ZNF766, RAC1, ZNF641, KDM6B, WARS, TSC22D2, KIF23, ETV3, TERF2, ESRP2, BIRC5, PGAM4, ZNF117, ALDOA, OTUB1, RBM22, PCNA, POLDIP2, SATB1, MGRN1, FOXK1, PRICKLE1, RNF4, PRICKLE4, AURKA, PURB, PARD6B, USP1, SHOC2, SMNDC1, EXOSC2, PURG, SLC35A2, IRX2, HNRNPA3, ANGPT2, WTAP, SPRYD4, CDC5L, USF3, LYRM4, NFIC, R3HDM4, TACC2, SSBP2, CAPS, ZNF695*	
Cytoplasm	0.001	0.117
GO:0005737	*BTG1, YWHAB, RTCB, ZDHHC5, IPP, ILK, FAM208B, STON2, CDC73, CDC14B, CHAF1B, PNP, SESN3, ADAMTS1, PPP6R3, PIM1, AKT1, RAC1, ZNF641, MAP2K4, WARS, PGAM1, TSC22D2, SLC30A4, LARP4B, DICER1, TERF2, IL17RB, SLC7A5, IMPACT, C1ORF116, MT1A, CTTN, RILPL1, CSDE1, HAT1, TBPL1, BIRC5, C19ORF24, RPL28, KPNB1, RBM22, OTUB1, TRUB2, PCNA, TUFT1, MGRN1, ARHGAP18, SPATA2, PIK3R1, RNF4, ZFP36L1, MTDH, PARD6B, ATXN1, RDH10, PPY, G3BP2, SHOC2, ZNF268, SMNDC1, EXOSC2, SPTBN2, RUNX1T1, SPAG9, TRAPPC2, HNRNPA3, SIVA1, FEM1A, KLHL21, DCDC2, CDC5L, PTK6, RPS28, GNL3L, EIF2S3, ID2, CASS4, MDM2, NF2, TACC2, SSBP2, BRWD1, PPIL4, CAPS, RPS23*	
Nucleoplasm	0.023	1
GO:0005654	*CDKN1A, PCNA, SATB1, RTCB, ILK, FAM208B, RNF4, CDC73, AURKA, CDC14B, CHAF1B, ATXN1, TRIM28, PPP6R3, USP1, AKT1, UBXN7, SHOC2, EXOSC2, ZNF641, RUNX1T1, KDM6B, SUPT16H, HNRNPA3, SIVA1, WTAP, UTP4, SMARCA5, CDC5L, KIF23, PTK6, TERF2, RPS28, HNRNPUL1, ESRP2, RILPL1, ID2, HAT1, MDM2, BIRC5, MDM4, HPSE, PPIL4, KPNB1, RBM22, RPS23*	
PCNA-p21 complex	0.024	1
GO:0070557	*CDKN1A, PCNA*	
Cytosol	0.024	1
GO:0005829	*CDKN1A, YWHAB, AMD1, ILK, IRS2, CHAF1B, PNP, PPP6R3, AP1S2, AKT1, CA6, RAC1, ATP6V1E1, EPHB4, MAP2K4, MYLIP, WARS, PGAM1, KIF23, LARP4B, WDR73, DICER1, SLC7A5, MT1A, RILPL1, PGAM4, BIRC5, ALDOA, RPL28, STEAP2, KPNB1, MGRN1, ARHGAP18, PRICKLE1, PIK3R1, AURKA, ZFP36L1, PARD6B, VPS53, G3BP2, PLCG2, EXOSC2, SPTBN2, RANBP2, SPAG9, TRAPPC2, NTRK2, PTK6, RPS28, EIF2S3, ID2, MDM2, RPS23*	

Identified using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

Supplementary Table S6. Significant correlation coefficients between plasma and urine microRNAs (total N=177 microRNAs).

miRNA	**Correlation**	***P*-value**
hsa-let-7a-3p	0.88	<.001
hsa-let-7a-5p/7c-5p	0.87	<.001
hsa-let-7b-5p	0.84	<.001
hsa-let-7d-3p	0.70	.01
hsa-let-7d-5p	0.70	.01
hsa-let-7e-5p	0.68	.01
hsa-let-7f-5p	0.68	.01
hsa-let-7g-5p	0.65	.02
hsa-let-7i-5p	0.65	.02
hsa-miR-1-3p	0.62	.03
hsa-miR-100-5p	0.61	.03
hsa-miR-101-3p	-0.61	.04

P-values were adjusted for multiple comparisons using Holm's method.

CHAPTER 7

Longitudinal epigenome-wide association studies of three male military cohorts reveal multiple CpG sites associated with post-traumatic stress disorder

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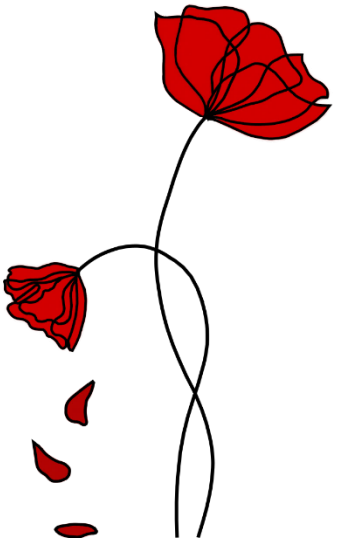


Abstract

Epigenetic mechanisms have been suggested to play a role in the development of post-traumatic stress disorder (PTSD). Here, blood-derived DNA methylation data (HumanMethylation450 BeadChip) collected prior to and following combat exposure in three cohorts of male military members were analyzed to assess whether DNA methylation profiles are associated with the development of PTSD. A total of 123 PTSD cases and 143 trauma-exposed controls were included in the analyses. The Psychiatric Genomics Consortium (PGC) PTSD EWAS QC pipeline was used on all cohorts, and results were combined using a sample size weighted meta-analysis in a two-stage design. In stage one, we jointly analyzed data of two new cohorts (N=126 and 78) for gene discovery, and sought to replicate significant findings in a third, previously published cohort (N=62) to assess the robustness of our results. In stage 2, we aimed to maximize power for gene discovery by combining all 3 cohorts in a meta-analysis. Stage 1 analyses identified four CpG sites in which, conditional on pre-deployment DNA methylation, post-deployment DNA methylation was significantly associated with PTSD status after epigenome-wide adjustment for multiple comparisons. The most significant (intergenic) CpG cg05656210 (p = 1.0 x 10^{-08}) was located on 5q31 and significantly replicated in the third cohort. In addition, 19 differentially methylated regions (DMRs) were identified, but failed replication. Stage 2 analyses identified 3 epigenome-wide significant CpGs, the intergenic CpG cg05656210 and two additional CpGs located in *MAD1L1* (cg12169700) and *HEXDC* (cg20756026). Cg12169700 had an underlying single nucleotide polymorphism (SNP) which was located within the same LD block as a recently identified PTSD-associated SNP in *MAD1L1*. Stage 2 analyses further identified 12 significant differential methylated regions (DMRs), one of which was located in *MAD1L1* and four were situated in the human leukocyte antigen (HLA) region. This study suggests that the development of combat-related PTSD is associated with distinct methylation patterns in several genomic positions and regions. Our most prominent findings suggest the involvement of the immune system through the HLA region and *HEXDC*, and *MAD1L1* which was previously associated with PTSD.

Keywords: EWAS, longitudinal, DNA methylation, meta-analysis, trauma, PTSD, epigenetics

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychiatric disorder that can develop following direct or indirect exposure to a potentially life-threatening traumatic incident. Symptoms include persistent re-experiencing of the trauma, avoidance behavior, hyperarousal and negative mood [1]. Although most individuals have the potential to withstand negative effects of trauma exposure on long-term mental health and recover promptly, some are more vulnerable and at increased risk of developing PTSD. Understanding the molecular and neurobiological underpinnings of this differential susceptibility is currently receiving considerable attention, and epigenetic mediation of environmental influences has been proposed as a potential key mechanism [2-4].

Several epigenome-wide association studies (EWAS) have aimed to identify differentially methylated CpGs in PTSD [5-8]. However, most of these studies are based on association analyses where methylation was assessed at a single time point (cross-sectional), with limited ability to adjust for confounding variables. Only one PTSD study to date reported longitudinal changes in methylation profiles across a period of combat exposure in order to capture changes in DNA methylation over time in relation to phenotypic changes [7].

Here, we followed a previously published two-stage design [9] where we first combined two longitudinal, US-based military cohorts in order to identify associations between post-deployment DNA methylation and PTSD symptoms, while accounting for differences in pre-deployment methylation levels. We then sought replication of our significant findings using the Prospective Research In Stress-related Military Operations (PRISMO) study. In the second stage, we combined all 3 cohorts in a meta-analysis. This 2-stage approach allows us to investigate the robustness of our findings through replication in stage 1, while increasing power for gene discovery by combining all studies in stage 2. In all cohorts, DNA methylation and phenotypic data was collected prior to and following a 4 to 7 month deployment period to an active ware zone in Iraq or Afghanistan. Of the significant CpGs in the second analysis stage, we assessed associations with nearby single nucleotide polymorphisms (SNPs) and examined correlations between blood and brain methylation status.

To the best of our knowledge, this is the largest study aimed at detecting methylation patterns associated with PTSD status while correcting for pre-trauma methylation levels. This longitudinal analysis permits us to more accurately capture DNA methylation patterns in PTSD while minimizing confounding due to intra-individual variability.

Materials and methods

Discovery datasets

Marine Resiliency Study

The Marine Resiliency Study (MRS) [10] is a prospective PTSD study of Marines and Navy personnel deployed to Iraq or Afghanistan. PTSD symptoms were assessed approximately one month before deployment, three and/or six months post-deployment using the CAPS and the PTSD Checklist (PCL) for DSM-IV. Biological samples including whole blood were collected at all time points. Information on smoking and alcohol use was collected on a self-report basis. Combat exposure was assessed approximately one week post-deployment using the Deployment Risk and Resilience Inventory (DRRI). A subset of 63 PTSD cases and 63 controls was selected for the methylation assays and inclusion in the present study. All subjects were free of a PTSD diagnosis at pre-deployment and had CAPS scores ≤ 25. After return from a ~7-months deployment period, blood samples from PTSD cases (following the DSM-IV full or partially stringent diagnosis [11, 12]) were selected either at the three- or the six-month follow-up visit, based on when these subjects had their highest recorded CAPS scores. Subsequently, controls were frequency matched to the selected cases for age, ancestry, and time of post-deployment visit. The study was approved by the institutional review boards of the University of California San Diego, VA San Diego Research Service, and Naval Health Research Center. All subjects provided informed consent.

Army STARRS

The Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS) is a prospective study among U.S. Army personnel gathering information on risk and resilience factors for suicidality and psychopathology [13]. All subjects completed a computerized version of the Composite International Diagnostic Interview screening scales (CIDI-SC) and the PCL6 screener for DSM-IV approximately 6 weeks before deployment to Afghanistan, and the PCL-C at one, two, and six months post-deployment. PTSD diagnosis was assigned using multiple imputation methods that relied on PCL and CIDI-SC data [14]. Information on trauma exposure was gathered from self-administered questions on childhood, adult, and military-related events. Information on smoking and alcohol use was collected on a self-report basis. Biological samples including whole blood were collected approximately 6 weeks before deployment and one month post-deployment. A subset of 31 cases and 47 controls were selected for methylation assays and for inclusion in this analysis. All subjects were free of a PTSD diagnosis at pre-

deployment. PTSD cases were selected based on their PTSD diagnosis at 6 months post-deployment. Controls were PTSD-free subjects matched on age, deployment stress and childhood adversity. The study procedures were approved by the Institutional Review Boards of all collaborating organizations. All subjects provided informed consent.

Replication dataset: PRISMO

Replication data was obtained from the PRISMO study, a prospective study of Dutch military soldiers deployed to Afghanistan [15, 16]. The severity of current PTSD symptoms was assessed using the Self-Report Inventory for PTSD (SRIP) and blood samples were collected approximately one month before and one and six months after deployment. Traumatic stress exposure during deployment to Afghanistan was assessed with a deployment experiences checklist. Information on smoking and alcohol use was collected on a self-report basis. A subset of 29 cases and 33 controls was selected for the methylation assays and inclusion in this analysis (see [7] for selection criteria). The study was approved by the ethical committee of the University Medical Center Utrecht, and was conducted in accordance with the Declaration of Helsinki. All subjects provided informed consent.

Quality control

In all cohorts, longitudinal whole blood DNA methylation levels were measured using the Illumina HumanMethylation450K BeadChip. The Psychiatric Genomics Consortium (PGC)-EWAS quality control pipeline was used on all three cohorts [5]. Briefly, samples were excluded when having a probe detection call rate <90% and an average intensity value <50% of the overall sample mean or <2,000 arbitrary units (AU). Individual probes with detection *p*-values >0.001 or those based on less than three beads were set to missing. Remaining probes were excluded when cross-reactivity occurred between autosomal and sex chromosomes. CpG sites with missing data for >10% of samples within cohorts were excluded (Supplementary Table S5). After filtering, the β-values reflecting methylation levels of individual cytosine residues were normalized to correct for differences between type I and type II probes using Beta Mixture Quantile Normalization (BMIQ) [17]. ComBat [18] was used to correct for remaining issues such as batch and plate effects. To account for differences in cell type composition between samples, proportions of CD8, CD4, NK, B cells, monocytes and granulocytes were estimated for each individual using their unique DNA methylation profiles. This was estimated using the estimatecellcounts function in minfi [19].

Statistical analysis

The normalized β-values were logit transformed to M-values which were used for linear regression analysis. Post-deployment DNA methylation was modeled as a function of post-deployment PTSD status while adjusting for pre-deployment DNA methylation, age, changes in CD4T, CD8T, NK, B cell, and monocyte cell proportions, and principal components (PCs) for ancestry. For MRS and Army STARRS, the PCs were derived from available GWAS and PCs 1-3 were included. For PRISMO, the method described by Barfield and colleagues [20] was used to derive PCs from the EWAS data and PCs 2-4 (see [5]) were included. HC3 standard errors were calculated using the sandwich R library [21]. Analyses were performed on each cohort independently and the obtained *p*-values were combined using a sample size weighted meta-analysis. Significance was declared at $p < 1.13 \times 10^{-7}$ after a stringent Bonferroni correction for 439,897 probes. Possible confounding effects of changes in smoking and alcohol use were assessed as a sensitivity analysis.

DMR analysis was performed on a set of 26,000 pre-defined gene regions within gene bodies, promoter regions, and CpG islands using the mCSEA version 1.2 package for R [22]. Regions were included when annotated to having at least 5 CpGs. For each study, EWAS *p*-values, methylation level values, and a phenotype and covariate data matrix were supplied as program inputs. P-values were derived using 100,000 permutations. A sample size weighted meta-analysis of DMRs was performed based on *z*-score transformations of permutation *p*-values. Significances of DMRs ($p < 1.92 \times 10^{-6}$) were derived based on a Bonferroni correction for the 26,000 tests performed. All positions and regions were in reference to GRCh37/hg19.

We considered replication as significant when the effect directions matched between the discovery and replication samples and the *p*-values held up to Bonferroni correction for the number of replications attempted (i.e. 4 for the DMPs, 19 for the DMRs) using a one-sided test.

Detecting associated genetic effects

Associations between baseline levels of methylation of each significant CpG from the second analysis stage and nearby SNPs (within 500 kilobases; kb) were assessed in the MRS dataset using PLINK [23] to detect the potential influence of genetic effects on DNA methylation. For a given CpG site, the SNP with the lowest *p*-value was carried forward as an additional covariate in the regression models as a sensitivity analysis. We used the UCSC genome browser tool (http://genome.ucsc.edu/) to identify if SNPs associated with

our CpGs influenced expression in other tissue types based on combined expression eQTL data from 44 tissues from GTEx v6 [24].

Blood-brain correlations

The Blood Brain DNA Methylation Comparison Tool (http://epigenetics.iop.kcl.ac.uk/bloodbrain/) was used to assess correlations between the methylation status of the top hits of the combined analyses in blood and brain [25]. This tool yields Pearson's correlation coefficients (*r*) and associated *p*-values for the association of the methylation status of individual CpG sites in blood and the prefrontal cortex, entorhinal cortex, superior temporal gyrus, and cerebellum.

Results

Cohorts

Three military cohorts were included in this study; the US-based MRS and Army STARRS, and the Dutch PRISMO study. Demographic and clinical characteristics of subjects from all three cohorts (total N subjects = 266) can be found in Table 1. All subjects were male and the majority were of European ancestry (N=211, 79%). Within each cohort, cases and controls did not differ significantly in terms of age. Pre-deployment PTSD symptoms were significantly different between cases and controls from MRS only, with cases scoring slightly higher on the Clinician Administered PTSD Scale (CAPS) as compared to controls (p=.002; Table 1). In MRS and Army STARRS, cases were exposed to more traumatic events as compared to controls (p<.001 for both cohorts).

Stage 1: MRS and Army STARRS

Data from MRS and Army STARRS were combined in order to identify CpG sites in which, conditional on baseline DNA methylation, post-deployment methylation was associated with PTSD status. Four genome-wide significant CpG sites (i.e. differentially methylated positions, DMPs) were identified using a Bonferroni threshold of $p = 1.13 \times 10^{-07}$ for the 450K EWAS array (Table 2). These sites were located near *SPRY4,* in *SDK1*, *CTRC* and *CDH15*. The direction of DNA methylation profiles associated with PTSD development was different for each site (Supplementary Figures S1-4). After Bonferroni correction for ~26,000 predefined regions, 19 differentially methylated regions (DMRs) were identified in which, conditional on baseline DNA methylation, post-deployment methylation was significantly associated with PTSD status (Table 3).

Replication in PRISMO

After Bonferroni correction for the 4 significant DMPs and when using a one-sided test, the association of one CpG site, the intergenic site cg05656210, was replicated in PRISMO (p=2.0 x 10^{-02}; Table 2). Both the discovery meta-analysis and replication analysis show decreased DNA methylation in association with PTSD status. None of the 19 significant DMRs were replicated in PRISMO (Table 3).

Table 1. Demographic and clinical characteristics of MRS, Army STARRS and PRISMO.

	Cases	**Controls**	***P*-value**	**Overall**
N				
MRS	63	63	-	126
Army STARRS	31	47	-	78
PRISMO	29	33	-	62
Age, mean (SD)				
MRS	22.15 (2.3)	22.36 (3.7)	.71	22.26 (3)
Army STARRS	23.5 (4.0)	24.6 (4.8)	.26	24.2 (4.4)
PRISMO	27.1 (9.9)	27.1 (8.7)	1.0	27.1 (9.0)
PTSD pre-deployment, mean (SD)				
MRS, CAPS	10.8 (7.5)	6.8 (6.5)	.002	8.8 (7)
Army STARRS, PCL-6	7.4 (2.6)	6.8 (2.0)	.40	7.0 (2.2)
PRISMO, SRIP	28.2 (4.0)	26.4 (4.0)	.10	27.2 (3.9)
PTSD post-deployment, mean (SD)				
MRS, CAPS	58.17 (13.5)	13.36 (6.1)	< .001	35.76 (9.8)
Army STARRS, PDL-C	52.7 (7.8)	25.8 (8.6)	< .001	36.5 (8.1)
PRISMO, SRIP	46.1 (8.7)	27.4 (5.1)	< .001	36.1 (6.5)
Combat exposure, mean (SD)				
MRS, DDRI	1.08 (0.8)	0.66 (0.4)	< .001	0.87 (0.6)
Army STARRS, PCL	9.4 (1.3)	7.9 (2.0)	< .001	8.5 (1.7)
PRISMO, DEC	8.5 (3.0)	7.2 (2.3)	.07	7.8 (2.5)
Ancestry, N (%)				
MRS				
- European	34 (53)	37 (59)		71 (56)
- African	5 (8)	5 (8)		10 (8)
- Other	24 (39)	21 (33)		45 (36)
Army STARRS				
- European	31 (100)	47 (100)		78 (100)
PRISMO				
- European	29 (100)	33 (100)		62 (100)

CAPS: Clinician-Administered PTSD Scale, PCL-6: PTSD Checklist – screener, SRIP: Self-Report Inventory for PTSD, PCL-C: PTSD Checklist – civilian version, DDRI: Deployment Risk and Resilience Inventory, DEC: deployment experiences checklist. SD: standard deviation. Each study used different scales for PTSD and combat exposure scores; the corresponding scales are included in the row names.

Table 2. Differentially methylated positions (DMPs) in MRS, Army STARRS and PRISMO.

				MRS			Army STARRS		
Probe	**Chr: position**	**Gene**	**Region**	**β**	**SE**	***P*-val**	**β**	**SE**	***P*-val**
cg05656210	5: 141660565	Interg	Interg	-0.37	0.15	1.6E-02	-0.58	**0.09**	6.1E-10
cg12169700	7: 1923695	*MAD1L1*	Body	-1.24	0.27	4.2E-06	-0.19	0.20	3.3E-01
cg20756026	17: 80394529	*HEXDC*	Body	-0.62	0.21	3.3E-03	-0.28	0.09	2.6E-03
cg16956686	7: 4304779	*SDK1*	Body	-0.19	0.04	3.6E-07	-0.13	**0.05**	7.1E-03
cg18917957	1: 15764093	*CTRC*	TSS1500	-0.34	0.08	2.3E-05	-0.26	**0.08**	4.9E-04
cg05901543	16: 89251975	*CDH15*	Body	-0.14	0.03	2.4E-08	-0.06	**0.03**	7.1E-02

SE: standard error. All positions and regions were in reference to GRCh37/hg19. Significance ($p < 1.13 \times 10^{-7}$) is indicated in bold. The asterisk indicates significance of replication after Bonferroni correction for four probes (one-sided z-test). The *p*-values for MRS, Army STARRS and the combined analyses are Bonferroni-corrected for ~450K CpG sites. In stage 1, MRS and Army STARRS were combined and PRISMO was used to replicate significant findings. In stage 2, all three studies were combined. The table is organized based on significances of the DMPs in the stage 2 meta-analysis. Chr: chromosome, interg: intergenic, p-val: p-value.

Table 2. Continued.

	Stage 1: MRS and Army STARRS		**Stage 1: Replication in PRISMO**			**Stage 2: Meta-analysis of 3 cohorts**	
Probe	**Z**	***P*-value**	**β**	**SE**	***P*-value**	**Z**	***P*-value**
cg05656210	-5.73	**1.0E-08**	-0.47	0.20	2.0E-02*	-6.14	**8.1E-10**
cg12169700	-4.22	2.4E-05	-0.64	0.14	4.3E-06	-5.91	**3.3E-09**
cg20756026	-4.17	3.0E-05	-0.37	0.09	2.6E-05	-5.69	**1.3E-08**
cg16956686	-5.67	**1.5E-08**	-0.04	0.09	6.3E-01	-5.20	2.0E-07
cg18917957	-5.48	**4.2E-08**	-0.06	0.13	6.4E-01	-5.03	5.0E-07
cg05901543	-5.50	**3.7E-08**	0.01	0.05	8.2E-01	-4.71	2.5E-06

SE: standard error. All positions and regions were in reference to GRCh37/hg19. Significance ($p < 1.13 \times 10^{-7}$) is indicated in bold. The asterisk indicates significance of replication after Bonferroni correction for four probes (one-sided z-test). The *p*-values for MRS, Army STARRS and the combined analyses are Bonferroni-corrected for ~450K CpG sites. In stage 1, MRS and Army STARRS were combined and PRISMO was used to replicate significant findings. In stage 2, all three studies were combined. The table is organized based on significances of the DMPs in the stage 2 meta-analysis.

Table 3. Differentially methylated regions (DMRs) in MRS, Army STARRS and PRISMO

			MRS		Army STARRS		Stage 1: MRS and Army STARRS		Stage 1: Replication in PRISMO		Stage 2: Meta-analysis of 3 cohorts	
Chr: # of probes	**Gene**	**Region**	**NES**	***P*-val**	**NES**	***P*-val**	**Z**	***P*-val**	**NES**	***P*- val**	**Z**	***P*-val**
6: 56	*HLA-DPB1*	Body	-2.08	3.45E-05	-1.98	8.06E-05	-5.69	**1.25E-08**	-1.05	3.51E-01	-5.43	**5.46E-08**
6: 17	*HLA-DBP1*	Island	-2.04	6.96E-05	-2.25	1.62E-04	-5.46	**4.80E-08**	-1.16	2.15E-01	-5.38	**7.49E-08**
21: 10	*KCNE1*	Island	-1.93	1.32E-04	-2.16	1.72E-04	-5.33	**9.99E-08**	-1.27	1.58E-01	-5.34	**8.93E-08**
21: 23	*KCNE1*	Prom	-1.93	9.45E-05	-2.0	3.42E-04	-5.28	**1.27E-07**	-1.22	1.91E-01	-5.26	**1.46E-07**
6: 34	*HLA-DRB1*	Body	-1.23	2.00E-01	-2.48	1.67E-05	-3.67	2.43E-04	-2.58	2.62E-05	-5.24	**1.58E-07**
7: 3	*MAD1L1*	Island	-2.22	1.81E-05	-2.12	1.72E-04	-5.69	**1.25E-08**	-0.81	7.38E-01	-5.15	**2.65E-07**
7: 10	*HOXA4*	Island	-2.21	1.73E-05	-1.98	3.26E-04	-5.6	**2.15E-08**	-0.84	7.46E-01	-5.06	**4.20E-07**
8: 9	*TRMT12*	Prom	-2.2	1.85E-05	-1.14	2.98E-01	-4.01	6.11E-05	-1.93	1.55E-03	-5.03	**4.69E-07**
6: 13	*HLA-DRB1*	Island	-1.25	1.79E-01	-2.49	1.68E-04	-3.38	7.17E-04	-2.52	2.59E-05	-4.99	**5.92E-07**
7: 24	*HOXA4*	Prom	-2.31	1.78E-05	-1.97	3.41E-04	-5.59	**2.31E-08**	-0.68	9.18E-01	-4.94	**7.71E-07**
6: 4	*SLC17A3*	Island	-1.96	9.39E-05	-1.50	4.91E-02	-4.29	1.81E-05	-1.63	3.04E-02	-4.8	**1.60E-06**
1: 5	*NTRK1*	Island	-1.51	5.29E-02	-1.78	3.74E-03	-3.31	9.20E-04	-2.12	1.16E-04	-4.76	**1.90E-06**
16: 121	*IFT140*	Body	-1.67	7.56E-04	-1.82	5.99E-05	-5.12	**2.92E-07**	-0.83	8.80E-01	-4.57	4.96E-06
17: 12	*MFSD6L*	Prom	-2.33	1.84E-05	-1.82	3.56E-03	-5.17	**2.36E-07**	1.03	4.10E-01	-4.52	6.33E-06
16: 8	*IFI140*	Island	-2.46	1.81E-05	-1.79	2.75E-03	-5.22	**1.78E-07**	0.69	8.80E-01	-4.5	6.79E-06
5: 5	*LRRC14B*	Island	-2.19	1.81E-05	-1.57	2.34E-02	-4.77	**1.83E-06**	-0.93	5.50E-01	-4.47	7.96E-06
6: 18	*KIF25*	Body	-2.13	5.45E-05	-1.79	4.11E-03	-4.94	**7.59E-07**	-0.62	9.50E-01	-4.36	1.30E-05
10: 5	*DIP2C*	Island	-2.22	3.54E-05	-1.98	5.05E-04	-5.4	**6.63E-08**	1.148	2.60E-01	-4.19	2.78E-05
1: 608	*PRDM16*	Body	-1.73	1.34E-05	-1.43	2.06E-04	-5.71	**1.09E-08**	1.168	8.00E-02	-4.15	3.25E-05
17: 7	*MFSD6L*	Island	-2.19	1.84E-05	-1.82	2.42E-03	-5.24	**1.59E-07**	0.684	8.70E-01	-4.13	3.57E-05
12: 6	*LINC00612*	Island	-2.27	1.84E-05	-2.36	1.75E-04	-5.69	**1.30E-08**	1.465	6.00E-02	-4.09	4.31E-05
12: 9	*LOC144571*	Prom	-2.27	1.85E-05	-2.16	1.81E-04	-5.68	**1.34E-08**	1.53	4.00E-02	-4	6.46E-05
11: 6	*SHANK2*	Island	-2.56	1.83E-05	-2.32	1.73E-04	-5.69	**1.28E-08**	1.752	6.00E-03	-3.67	2.45E-04
17: 3	*TNRC63*	Island	-2.03	9.29E-05	-2.05	1.78E-04	-5.39	**7.07E-08**	1.693	1.10E-02	-3.49	4.82E-04

Chr: chromosome, NES: normalized effect score, Prom: Promoter. All positions and regions were in reference to GRCh37/hg19. Significance is indicated in bold. The *p*-values for MRS, Army STARRS and the combined analyses are Bonferroni-corrected for ~26K DMRs. In stage 1, MRS and Army STARRS were combined and PRISMO was used to replicate significant findings. In stage 2, all three studies were combined. The table is organized based on significances of the DMRs in the stage 2 meta-analysis. Start/stop sites were removed due to space constraints. The full table can be found here: 10.1186/s13148-019-0798-7 (doi).

Stage 2: Meta-analysis of MRS, Army STARRS and PRISMO

When combining MRS, Army STARRS and PRISMO, the DNA methylation profiles of three CpG sites were significantly associated with post-deployment PTSD status (Table 2, Figure 1). The intergenic CpG that replicated in PRISMO remained the most significant (Z= -6.14, p= 8.1 x 10^{-10}; Supplementary Figure S1). The other sites were located within the gene body regions of *MAD1L1* and *HEXDC* (Supplementary Figures S5, S6, respectively). Sensitivity analyses for the potentially confounding effects of smoking and alcohol use did not substantially affect these results (Supplementary Table S1). Furthermore, 12 DMRs were identified (Supplementary Figures S7-18, Figure 1), 7 of which were also significant in stage 1, and 4 were located within the human leukocyte antigen (HLA) region(Table 3).

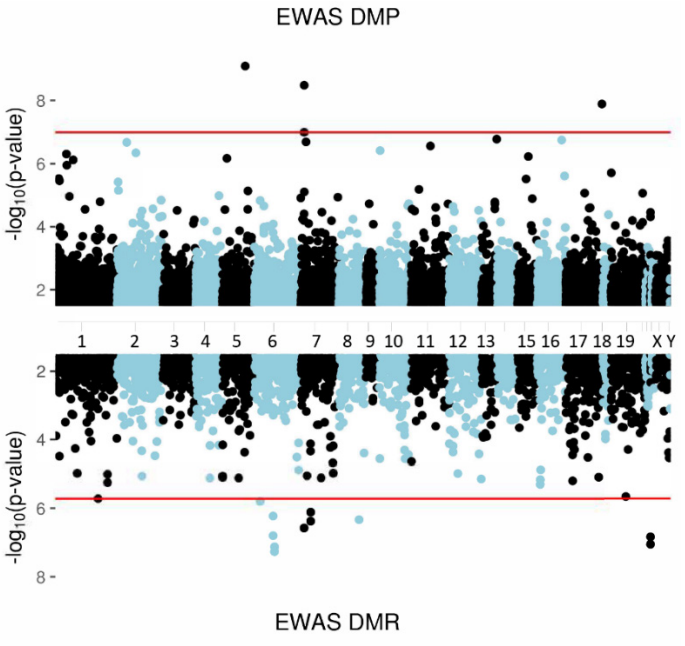


Figure 1. Manhattan plot showing the stage 2 meta-analysis across 3 epigenome-wide association studies (MRS, Army STARRS, PRISMO). The upper part shows the 3 significant differentially methylated positions (DMPs) while the lower part shows the 12 significant differentially methylated regions (DMRs). Red lines indicate significance thresholds after Bonferroni corrections for ~485,000 (top) and 26,000 (bottom) comparisons.

Genetic effects

Using MRS data on gene expression, genetic effects on DNA methylation levels of the significant DMPs were assessed by testing for associations with SNPs within 500kb of the DMPs. All DMPs had significantly associated SNPs which explained approximately 80% of the variation in methylation ($p < 2 \times 10^{-16}$) and were located within 1bp of their respective CpG sites (Supplementary Table S2). Adjusting for genotypes in the main model to assess the impact of SNPs on the association between DNA methylation and PTSD, did not substantially affect the observed findings (Supplementary Table S3).

Blood-brain correlations of PTSD-associated CpGs

Blood-brain correlations of methylation levels of the significant stage 2 DMPs were examined using a publicly available database [25]. For all three DMPs, blood DNA methylation levels correlated strongly with those in the prefrontal cortex, entorhinal cortex, superior temporal gyrus, and cerebellum ($r \geq 0.93$ for all sites; p-values ranging between 1.48×10^{-32} and 5.32×10^{-72}; Supplementary Table S4, Supplementary Figure S19 for cg05656210).

Discussion

Exposure to trauma is a prerequisite for the development of PTSD, yet not all individuals develop PTSD following trauma [26]. So far, the underlying biological mechanisms of this differential susceptibility have not been fully identified and even the largest PTSD genome-wide association studies (GWAS) to date explain only a small proportion of the disease liability [27, 28]. Epigenetic mechanisms have been studied as one potential mechanism, but most association studies used cross-sectional designs, which render it impossible to assess dynamic fluctuations in methylation levels over a period of trauma exposure. Here, we use a longitudinal design to investigate associations of DNA methylation with post-deployment PTSD status across very similar military cohorts deployed to combat in Iraq or Afghanistan. We started by combining the US-based MRS and Army STARRS cohorts and sought replication using the previously published Dutch PRISMO study [7]. To maximize power for new discoveries, we also performed a meta-analysis across all 3 cohorts. The first analysis stage revealed four genome-wide significant DMPs and 19 DMRs which were linked to PTSD status. One of these DMPs replicated in PRISMO. In the second stage, a meta-analysis of all three studies revealed that the replicating DMP and 7 DMRs remained significant, and 2 additional DMPs and 12 DMRs were identified.

The replicating DMP at cg05656210 remained the top-ranked significant marker in the second analysis stage. This CpG site is an intergenic site annotated near *SPRY4*. SPRY4 is a member of the Sprouty proteins which are mainly involved in inhibiting receptor tyrosine kinase (RTK) signaling [29]. Upon activation by growth factor ligands, RTK signaling has a wide variety of downstream effects ranging from the regulation of cell proliferation and differentiation to the modulation of cellular metabolism [30]. One particular receptor involved in RTK signaling, i.e. receptor tyrosine kinase B (TrkB), and its main ligand, brain-derived neurotrophic factor (BDNF), have repeatedly been shown to be affected in stress-related disorders such as depression [31]. Consistently, two independent studies reported decreased mRNA levels of BDNF and TrkB in the prefrontal cortex and hippocampus of individuals who committed suicide as compared to healthy control subjects [32, 33]. Moreover, SPRY4-IT1, a long non-coding RNA derived from the second intron of *SPRY4* [34], has been shown to interact with *SKA2* [35], a gene that has been suggested to be a promising biomarker for suicidal behavior [36, 37], stress susceptibility and stress-related disorders such as PTSD [37-39]. Finally, *SPRY4* was previously found differentially methylated in the blood of patients diagnosed with schizophrenia [40]. Together, these results suggest that alterations within *SPRY4* could

contribute to psychiatric disorders such as depression, PTSD and schizophrenia and potentially play a role in suicidal behavior. The question as to whether and how the identified DMP influences the expression of *SPRY4* and is involved in these phenotypes, remains to be answered.

The second top significant probe, cg12169700, and one DMR are located within *MAD1L1*. MAD1L1 is part of the mitotic spindle-assembly checkpoint (SAC) which monitors the proper attachment of chromosomes to the microtubule spindle apparatus, delays the start of anaphase until all chromosomes are properly attached, and in doing so, ensures correct chromosome separation [41, 42]. Malfunctions of the MAD1L1 protein could therefore contribute to aneuploidy and chromosomal instability. Specific SNPs within this gene have previously been associated with bipolar disorder [43, 44], schizophrenia [44-46] and depression [47]. Interestingly, *MAD1L1* was recently identified in a PTSD GWAS of the Million Veteran Program (MVP) [48]. The SNP that underlies cg12169700, rs11761270, is located within the same large linkage disequilibrium (LD) block as the MVP finding. Previous findings further showed that blood levels of *MAD1L1* were decreased in highly stress-susceptible individuals [49]. Together, these findings suggest that specific profiles within *MAD1L1* may be regarded as a risk factor for PTSD in addition to several other psychiatric disorders [50]. However, the underlying mechanisms through which such disturbances could contribute to psychiatric disorders warrant further research.

The third CpG site is located in *HEXDC* which to date has no known implications in psychiatric disorders. Although the biological functions of its product, hexosaminidase D, remain largely unknown, it is believed to be a glycosidase and previous studies found associations with rheumatoid arthritis [51, 52]. Interestingly, recent studies found significant pleiotropy between rheumatoid arthritis, PTSD [53], and schizophrenia [54]. Other studies found associations between PTSD symptoms and rheumatoid arthritis in a twin population [55] and in an epidemiological study of military veterans [56]. This suggested link between mental disorders and immune-related processes is discussed more in detail below. The DMP of *HEXDC* was located directly adjacent to rs4789774, a known expression quantitative trait locus (eQTL) that regulates the expression of *HEXDC* in the human brain cortex and of *NARF* and *NARF-IT1* in a number of tissue types including blood (http://genome.ucsc.edu/).

Twelve significant DMRs were found in the second phase of the analysis. Our strongest finding was in the HLA region, a gene-dense region which contains over 200 genes that encode human leukocyte antigen complex proteins in charge of presenting peptide

antigens to trigger immune reactions, among other (non-)immune functions [57]. Their non-immunological roles include involvement in processes such as neurodevelopment, synaptic plasticity, learning, memory and stress reactivity [58, 59]. It is therefore not entirely unexpected that several epigenetic modifications and genetic variants within this region have repeatedly been found associated to neuropsychiatric disorders such as schizophrenia and bipolar disorder (recently reviewed in [60]). Along with our *HEXDC* finding, these observations further enhance the existing notion that immune factors play an important role and should continue to be studied in relation to mental disorders such as PTSD [61]. Although it is now clear that immune imbalances are present in PTSD, questions related to causality and further implications for prevention strategies and treatment options largely remain to be answered.

Follow-up analyses were done using the significant DMPs from the stage 2 meta-analysis only. The discovery that methylation levels at the top three PTSD-associated CpGs were highly associated with the genotype of nearby SNPs, led us to question whether the associations between methylation and PTSD status were mainly driven by genotype. However, direct adjustment for genotype in a sensitivity analysis did not attenuate the associations between DNA methylation and PTSD status and our current sample size limits our ability to conduct analyses specific to genotype strata to further investigate interaction effects between SNPs and methylation.

Since the analyzed DNA methylation was derived from peripheral blood, we further examined correlations between blood and several brain regions, i.e. the prefrontal cortex, the entorhinal cortex, superior temporal gyrus and cerebellum. The results indicate that blood-brain correlations of all top DMPs were strong for all four brain regions suggesting that these findings could potentially also be relevant for tissues other than blood. Assessing these correlations is relevant when dealing with disorders such as PTSD which are characterized by several alterations within the brain but for which direct accessibility to human tissue is limited. However, these and similar findings will need to be confirmed using postmortem brain tissue and their precise role in PTSD development will need to be investigated further.

The main limitation of the present study is its small sample size, which likely captures only a fraction of all implicated CpGs and renders additional analyses such as pathway and network analyses underpowered. It further needs to be emphasized that this study used data generated with Illumina's 450K arrays, which only assess a subset of all CpG sites. Next, although examining blood-derived DNA methylation is informative when seeking

relatively easily accessible biomarkers, follow-up studies are needed in order to assess these methylation patterns within the tissue of interest, i.e. the brain. Another important limitation of the study is the fact that individuals who developed PTSD were exposed to significantly more traumatic events than control subjects in MRS and Army STARRS. One way to address these differences would be to include trauma exposure as a covariate. However, given the high correlation between trauma exposure and PTSD, this would likely diminish the association between methylation changes and PTSD. Therefore, we do acknowledge that our analyses may include associations with trauma exposure in addition to PTSD. Next, the limited replication of findings from the US cohorts in the Dutch PRISMO study, and vice versa [7], may point to type I errors, or be partially due to small sample sizes and/or heterogeneity in study designs, study environments, and potential confounders such as immune status or medication use. For example, findings reported from PRISMO [7] point towards the involvement of DMPs and DMRs located within *ZFP57, RNF39 and HIST1H2APS2,* which were not identified in our analyses. However, these genes are located within the HLA region, a region which we did identify in our analyses. Furthermore, whereas the PRISMO study is entirely based on subjects of European ancestry, the MRS and Army STARRS include more ancestral diversity. Genes in the HLA region tend to be highly polymorphic, particularly with respect to ancestral background [62]. Thus, it is possible that this heterogeneity may contribute to the lack of replication. Further analyses should be ancestry-specific, once sample sizes are adequate. Moreover, at this stage it is unclear whether the identified differential methylation patterns in PTSD cases have any functional consequences. Although they may influence gene expression, the current dataset has limited power to establish causality. In order to make claims regarding causation, performing functional studies *in vitro* and using animal studies are needed in order to unravel precise biological mechanisms. Finally, to maximize power for discovery, the present cohorts were chosen to be highly similar with regards to demographics, types of trauma, and time since trauma exposure. Thus, the degree to which these findings on active duty, predominantly European-ancestry military men, may generalize to females, civilians, or other ancestries, is unclear.

In summary, this is the largest study to date using both baseline and post-deployment DNA methylation levels to assess associations with combat-related PTSD. Our observations point towards the implication of biologically interesting genes such as the HLA region which is involved in immune-related processes, *HEXDC* which also has been suggested to play a role in immunity, and *MAD1L1*, a PTSD-related gene recently identified in the large MVP. These findings strengthen the notion that specific DNA methylation profiles are involved in combat-related PTSD. Larger longitudinal studies and

integrative efforts are now needed to build upon these preliminary findings in order to understand their functional consequences and integrate them more broadly into our current understanding of the (epi)genomic basis of PTSD.

References

1. Association, A.P., *Diagnostic and statistical manual of mental disorders (5th ed.)*. 2013, Arlington, VA: American Psychiatric Publishing.
2. Nievergelt, C.M., et al., *Genomic Approaches to Posttraumatic Stress Disorder: The Psychiatric Genomic Consortium Initiative.* Biol Psychiatry, 2018. **83**(10): p. 831-839.
3. Daskalakis, N.P., et al., *Recent Genetics and Epigenetics Approaches to PTSD.* Curr Psychiatry Rep, 2018. **20**(5): p. 30.
4. Sheerin, C.M., et al., *The Genetics and Epigenetics of PTSD: Overview, Recent Advances, and Future Directions.* Curr Opin Psychol, 2017. **14**: p. 5-11.
5. Ratanatharathorn, A., et al., *Epigenome-wide association of PTSD from heterogeneous cohorts with a common multi-site analysis pipeline.* Am J Med Genet B Neuropsychiatr Genet, 2017. **174**(6): p. 619-630.
6. Kuan, P.F., et al., *An epigenome-wide DNA methylation study of PTSD and depression in World Trade Center responders.* Transl Psychiatry, 2017. **7**(6): p. e1158.
7. Rutten, B.P.F., et al., *Longitudinal analyses of the DNA methylome in deployed military servicemen identify susceptibility loci for post-traumatic stress disorder.* Mol Psychiatry, 2018. **23**(5): p. 1145-1156.
8. Smith, A.K., et al., *Differential immune system DNA methylation and cytokine regulation in post-traumatic stress disorder.* Am J Med Genet B Neuropsychiatr Genet, 2011. **156B**(6): p. 700-8.
9. Story Jovanova, O., et al., *DNA Methylation Signatures of Depressive Symptoms in Middle-aged and Elderly Persons: Meta-analysis of Multiethnic Epigenome-wide Studies.* JAMA Psychiatry, 2018. **75**(9): p. 949-959.
10. Baker, D.G., et al., *Predictors of risk and resilience for posttraumatic stress disorder among ground combat Marines: methods of the Marine Resiliency Study.* Prev Chronic Dis, 2012. **9**: p. E97.
11. Sher, L., *Recognizing post-traumatic stress disorder.* QJM: An International Journal of Medicine, 2004. **97**(1): p. 1-5.
12. Smith, A.K., et al., *Epigenome-wide meta-analysis of PTSD across 10 military and civilian cohorts identifies novel methylation loci.* bioRxiv, 2019: p. 585109.
13. Ursano, R.J., et al., *The Army study to assess risk and resilience in servicemembers (Army STARRS).* Psychiatry, 2014. **77**(2): p. 107-19.
14. Kessler, R.C., et al., *Clinical reappraisal of the Composite International Diagnostic Interview Screening Scales (CIDI-SC) in the Army Study to Assess Risk and Resilience in Servicemembers (Army STARRS).* Int J Methods Psychiatr Res, 2013. **22**(4): p. 303-21.
15. Reijnen, A., et al., *Prevalence of mental health symptoms in Dutch military personnel returning from deployment to Afghanistan: a 2-year longitudinal analysis.* Eur Psychiatry, 2015. **30**(2): p. 341-6.
16. Eekhout, I., et al., *Post-traumatic stress symptoms 5 years after military deployment to Afghanistan: an observational cohort study.* Lancet Psychiatry, 2016. **3**(1): p. 58-64.
17. Teschendorff, A.E., et al., *A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data.* Bioinformatics, 2013. **29**(2): p. 189-96.
18. Johnson, W.E., C. Li, and A. Rabinovic, *Adjusting batch effects in microarray*

expression data using empirical Bayes methods. Biostatistics, 2007. **8**(1): p. 118-27.
19. Houseman, E.A., et al., *DNA methylation arrays as surrogate measures of cell mixture distribution.* BMC bioinformatics, 2012. **13**: p. 86-86.
20. Barfield, R.T., et al., *Accounting for population stratification in DNA methylation studies.* Genet Epidemiol, 2014. **38**(3): p. 231-41.
21. Zeileis, A., *Econometric Computing with HC and HAC Covariance Matrix Estimators.* Journal of Statistical Software, 2004. **11**(10).
22. Martorell-Marugan, J., V. Gonzalez-Rumayor, and P. Carmona-Saez, *mCSEA: Detecting subtle differentially methylated regions.* 2018: p. 293381.
23. Purcell, S., et al., *PLINK: a tool set for whole-genome association and population-based linkage analyses.* American journal of human genetics, 2007. **81**(3): p. 559-575.
24. Consortium, G.T., *The Genotype-Tissue Expression (GTEx) project.* Nature genetics, 2013. **45**(6): p. 580-585.
25. Hannon, E., et al., *Interindividual methylomic variation across blood, cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric phenotypes.* Epigenetics, 2015. **10**(11): p. 1024-32.
26. Yehuda, R., et al., *Post-traumatic stress disorder.* Nat Rev Dis Primers, 2015. **1**: p. 15057.
27. Gelernter, J., et al., *Genome-wide association study of post-traumatic stress disorder reexperiencing symptoms in >165,000 US veterans.* Nature Neuroscience, 2019. **22**(9): p. 1394-1401.
28. Nievergelt, C.M., et al., *International meta-analysis of PTSD genome-wide association studies identifies sex- and ancestry-specific genetic risk loci.* Nature Communications, 2019. **10**(1): p. 4558.
29. Mason, J.M., et al., *Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling.* Trends Cell Biol, 2006. **16**(1): p. 45-54.
30. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases.* Cell, 2010. **141**(7): p. 1117-1134.
31. Zhang, J.C., W. Yao, and K. Hashimoto, *Brain-derived Neurotrophic Factor (BDNF)-TrkB Signaling in Inflammation-related Depression and Potential Therapeutic Targets.* Curr Neuropharmacol, 2016. **14**(7): p. 721-31.
32. Dwivedi, Y., et al., *Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects.* Arch Gen Psychiatry, 2003. **60**(8): p. 804-15.
33. Pandey, G.N., et al., *Brain-derived neurotrophic factor and tyrosine kinase B receptor signalling in post-mortem brain of teenage suicide victims.* Int J Neuropsychopharmacol, 2008. **11**(8): p. 1047-61.
34. Khaitan, D., et al., *The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion.* Cancer Res, 2011. **71**(11): p. 3852-62.
35. He, X.J., et al., *Long non-coding RNA SPRY4-IT1 promotes the proliferation and invasion of U251 cells through upregulation of SKA2.* Oncol Lett, 2018. **15**(3): p. 3977-3984.
36. Guintivano, J., et al., *Identification and replication of a combined epigenetic and genetic biomarker predicting suicide and suicidal behaviors.* Am J Psychiatry, 2014. **171**(12): p. 1287-96.
37. Kaminsky, Z., et al., *Epigenetic and genetic variation at SKA2 predict suicidal behavior and post-traumatic*

stress disorder. Transl Psychiatry, 2015. **5**: p. e627.
38. Boks, M.P., et al., *SKA2 Methylation is Involved in Cortisol Stress Reactivity and Predicts the Development of Post-Traumatic Stress Disorder (PTSD) After Military Deployment*. Neuropsychopharmacology, 2016. **41**(5): p. 1350-6.
39. Sadeh, N., et al., *SKA2 methylation is associated with decreased prefrontal cortical thickness and greater PTSD severity among trauma-exposed veterans*. Mol Psychiatry, 2016. **21**(3): p. 357-63.
40. Zaharieva, I., et al., *Association study in the 5q31-32 linkage region for schizophrenia using pooled DNA genotyping*. BMC Psychiatry, 2008. **8**: p. 11.
41. Musacchio, A. and E.D. Salmon, *The spindle-assembly checkpoint in space and time*. Nat Rev Mol Cell Biol, 2007. **8**(5): p. 379-93.
42. Bolanos-Garcia, V.M., *Formation of multiprotein assemblies in the nucleus: the spindle assembly checkpoint*. Int Rev Cell Mol Biol, 2014. **307**: p. 151-74.
43. Trost, S., et al., *Investigating the Impact of a Genome-Wide Supported Bipolar Risk Variant of MAD1L1 on the Human Reward System*. Neuropsychopharmacology, 2016. **41**(11): p. 2679-87.
44. Zhao, L., et al., *Replicated associations of FADS1, MAD1L1, and a rare variant at 10q26.13 with bipolar disorder in Chinese population*. Transl Psychiatry, 2018. **8**(1): p. 270.
45. Ripke, S., et al., *Genome-wide association analysis identifies 13 new risk loci for schizophrenia*. Nat Genet, 2013. **45**(10): p. 1150-9.
46. Su, L., et al., *Genetic association of GWAS-supported MAD1L1 gene polymorphism rs12666575 with schizophrenia susceptibility in a Chinese population*. Neurosci Lett, 2016. **610**: p. 98-103.
47. Wray, N.R., et al., *Genome-wide association analyses identify 44 risk variants and refine the genetic architecture of major depression*. Nat Genet, 2018. **50**(5): p. 668-681.
48. Gelernter J., e.a., *Genome-wide association study of posttraumatic stress disorder (PTSD) re-experiencing symptoms in >165,000 US veterans*. Nature Neuroscience, 2019. **In press**.
49. Le-Niculescu, H., et al., *Towards precision medicine for stress disorders: diagnostic biomarkers and targeted drugs*. Molecular Psychiatry, 2019.
50. Levey, D.F., et al., *Reproducible Risk Loci and Psychiatric Comorbidities in Anxiety: Results from ~200,000 Million Veteran Program Participants*. bioRxiv, 2019: p. 540245.
51. Pasztoi, M., et al., *The recently identified hexosaminidase D enzyme substantially contributes to the elevated hexosaminidase activity in rheumatoid arthritis*. Immunol Lett, 2013. **149**(1-2): p. 71-6.
52. Alteen, M.G., et al., *Mechanism of Human Nucleocytoplasmic Hexosaminidase D*. Biochemistry, 2016. **55**(19): p. 2735-47.
53. Stein, M.B., et al., *Genome-wide Association Studies of Posttraumatic Stress Disorder in 2 Cohorts of US Army Soldiers*. JAMA psychiatry, 2016. **73**(7): p. 695-704.
54. Wang, Q., et al., *Pervasive pleiotropy between psychiatric disorders and immune disorders revealed by integrative analysis of multiple GWAS*. Human genetics, 2015. **134**(11-12): p. 1195-1209.
55. Boscarino, J.A., C.W. Forsberg, and J. Goldberg, *A Twin Study of the Association Between PTSD Symptoms and Rheumatoid Arthritis*. 2010. **72**(5): p. 481-486.

56. O'Donovan, A., et al., *Elevated risk for autoimmune disorders in iraq and afghanistan veterans with posttraumatic stress disorder.* Biological psychiatry, 2015. **77**(4): p. 365-374.
57. Beck, S. and J. Trowsdale, *The Human Major Histocompatibility Complex: Lessons from the DNA Sequence.* Annual Review of Genomics and Human Genetics, 2000. **1**(1): p. 117-137.
58. Huh, G.S., et al., *Functional requirement for class I MHC in CNS development and plasticity.* Science (New York, N.Y.), 2000. **290**(5499): p. 2155-2159.
59. Sankar, A., R.N. MacKenzie, and J.A. Foster, *Loss of class I MHC function alters behavior and stress reactivity.* Journal of Neuroimmunology, 2012. **244**(1): p. 8-15.
60. Debnath, M., et al., *The MHC/HLA Gene Complex in Major Psychiatric Disorders: Emerging Roles and Implications.* Current Behavioral Neuroscience Reports, 2018. **5**(2): p. 179-188.
61. Wang, Z., B. Caughron, and M.R.I. Young, *Posttraumatic Stress Disorder: An Immunological Disorder?* Frontiers in psychiatry, 2017. **8**: p. 222-222.
62. Katrinli, S., et al., *Association of HLA locus alleles with posttraumatic stress disorder.* Brain Behav Immun, 2019. **81**: p. 655-658.

Supplementary material

Supplementary Table S1. Differentially methylated positions (DMPs) in MRS, Army STARRS and PRISMO with and without corrections for smoking status and alcohol use.

		Stage 1: MRS and Army STARRS		**Stage 1: Replication in PRISMO**			**Stage 2: Meta-analysis of 3 cohorts**		**Meta-analysis of 3 cohorts, corrected for smoking and alcohol use**	
Probe	**Gene**	**Z**	***P*-val**	**β**	**SE**	***P*-val**	**Z**	***P*-val**	**Z**	***P*-value**
cg05656210	*SPRY4*	-5.73	**1.0E-08**	-0.47	0.20	**2.0E-02**	-6.14	**8.1E-10**	-6.50	**1.9E-10**
cg12169700	*MAD1L1*	-4.22	2.4E-05	-0.64	0.14	4.3E-06	-5.91	**3.3E-09**	-5.00	**6.1E-09**
cg20756026	*HEXDC*	-4.17	3.0E-05	-0.37	0.09	2.6E-05	-5.69	**1.3E-08**	-5.93	**4.5E-10**
cg16956686	*SDK1*	-5.67	**1.5E-08**	-0.04	0.09	6.3E-01	-5.20	2.0E-07	-5.13	4.4E-08
cg18917957	*CTRC*	-5.48	**4.2E-08**	-0.06	0.13	6.4E-01	-5.03	5.0E-07	-5.33	1.9E-07
cg05901543	*CDH15*	-5.50	**3.7E-08**	0.01	0.05	8.2E-01	-4.71	2.5E-06	-4.40	1.3E-06

SE: standard error. All positions and regions were in reference to GRCh37/hg19. Significance ($p < 1.13 \times 10^{-7}$) is indicated in bold. The significant *p*-value in PRISMO indicates significance of replication after Bonferroni correction for four probes (one-sided z-test). The *p*-values for MRS, Army STARRS and the combined analyses are Bonferroni-corrected for ~450K CpG sites. In stage 1, MRS and Army STARRS were combined and PRISMO was used to replicate significant findings. In stage 2, all three studies were combined. The table is organized based on significances of the DMPs in the stage 2 meta-analysis (without correction for smoking and alcohol).

Supplementary Table S2. SNPs within 500 bps upstream or downstream of the significant DMPs.

Probe (CpG)	**Chr: position**	**SNP ID**	**Strand**	**Distance US or DS**
cg05656210	5: 141660565	rs4998914	F	0
cg12169700	7: 1923695	rs11761270	F	0
cg20756026	17: 80394529	rs4789774	R	1 DS

Chr: chromosome, SNP: single nucleotide polymorphism, F: forward, R: reverse, US: upstream, DS: downstream

Supplementary Table S3. Differentially methylated positions (DMPs) in MRS with and without correction for main associated SNPs.

	Without SNPs			**With SNPs**		
	β	**SE**	***P*-value**	**β**	**SE**	***P*-value**
cg05656210	-0.37	0.15	1.6E-02	-0.35	0.15	2.11E-02
cg12169700	-1.24	0.27	4.2E-06	-1.29	0.22	6.46E-08
cg20756026	-0.62	0.21	3.3E-03	-0.47	0.18	9.11E-03

SE: standard error. Associated SNPs were rs7703928, rs11761270 and rs4789774, respectively.

Supplementary Table S4. Correlations between blood and brain methylation levels for the top CpG sites.

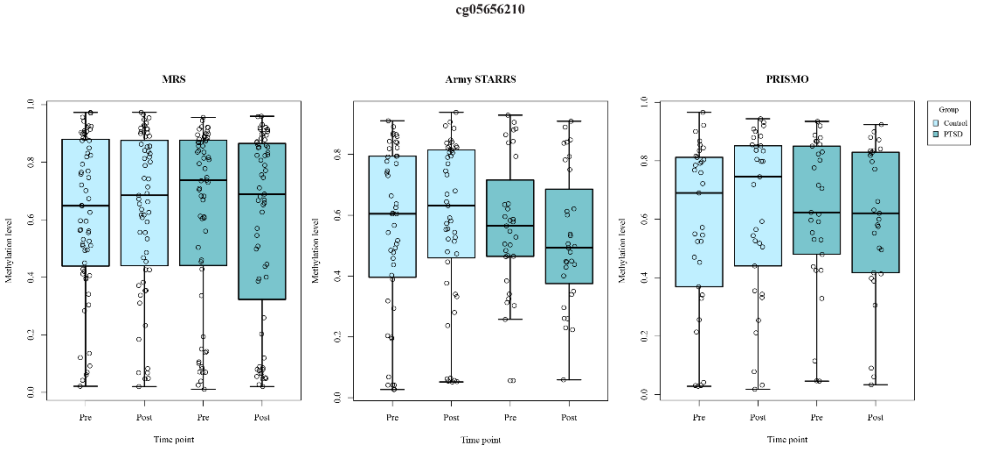
CpG	Brain region							
	PFC		EC		STG		CER	
	r	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
cg05656210	0.99	7.54E-58	0.99	8.5E-55	0.99	2.63E-60	0.99	1.57E-57
cg12169700	0.99	1.16E-62	0.98	1.32E-51	0.99	2.58E-65	0.93	1.48E-32
cg20756026	0.99	1.23E-68	0.99	1.45E-67	0.99	5.32E-72	0.99	4.64E-65

PFC: prefrontal cortex, EC: entorhinal cortex, STG: superior temporal gyrus, CER: cerebellum, r= Pearson correlation coefficient. Derived from http://epigenetics.essex.ac.uk/bloodbrain/.

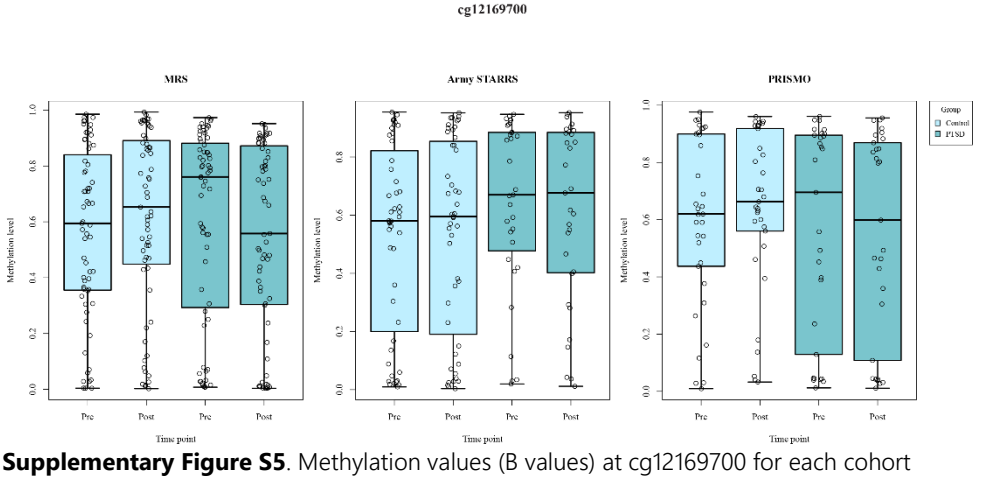
Supplementary Table S5. Number of probes and samples removed at each stage of the quality control (QC) pipeline

Probes QC	MRS	PRISMO	Army STARRS
Starting N probes	485,512	485,512	485,512
Removal of > 10% missingness	678	377	441
Removal of Cross-hybridizing probes	29,233	29,233	29,233
Final N probes	455,601	455,902	455,838
Samples QC			
Starting N samples	258	124	172
Low sample intensity	2	0	16
Sample mismatch	4	0	0
Final N samples	252 (126 pairs)	124 (62 pairs)	156 (78 pairs)

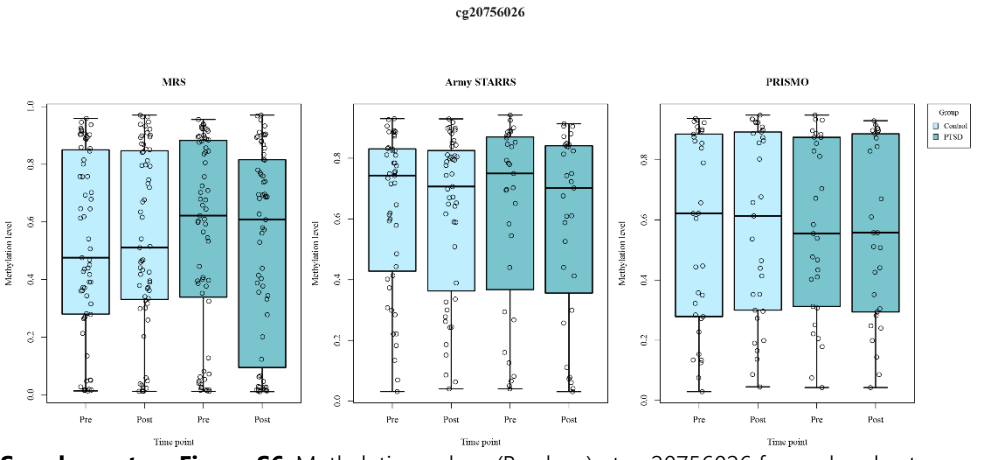
Given the large number of Supplementary Figures belonging to this chapter, only the most relevant figures are presented here. Remaining figures can be found here: 13148_2019_798_MOESM1_ESM.docx



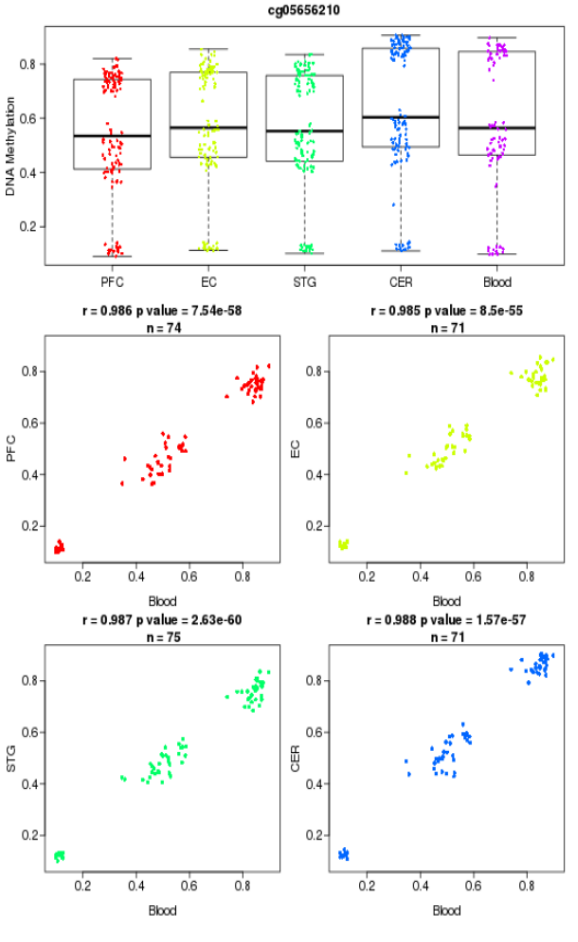
Supplementary Figure S1. Methylation values (B values) at cg05656210 for each cohort separately. Pre: pre-deployment, post: post-deployment.



Supplementary Figure S5. Methylation values (B values) at cg12169700 for each cohort separately. Pre: pre-deployment, post: post-deployment.



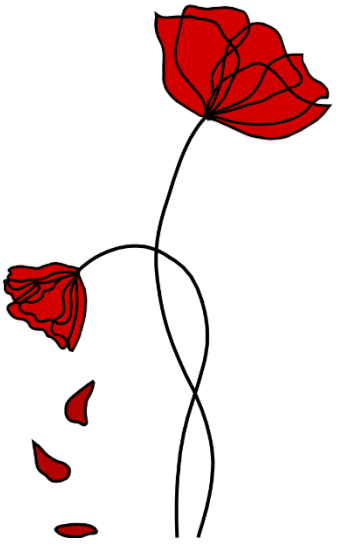
Supplementary Figure S6. Methylation values (B values) at cg20756026 for each cohort separately. Pre: pre-deployment, post: post-deployment.



Supplementary Figure S19. Example of blood-brain correlations of methylation levels in cg05656210. PFC: prefrontal cortex, EC: entorhinal cortex, STG: superior temporal gyrus, CER: cerebellum. This figure was retrieved from http://epigenetics.iop.kcl.ac.uk/bloodbrain/.

CHAPTER 8

General discussion



This thesis builds upon the available literature by highlighting several epigenetic processes associated with post-traumatic stress disorder (PTSD). In doing so, it provides deeper insights into potentially pathological mechanisms that characterize this disorder. The first part of this final chapter will be centered around the four main research questions which were presented in the introduction. This will be followed by an overview of the main limitations of the different chapters and insights into future perspectives.

Research question 1: What is known regarding resilience and traumatic stress and how can this be valuable for PTSD research?

As discussed in **Chapter 2**, resilience refers to a dynamic process that enables one to successfully overcome a stressful or traumatic experience. Resilience research aims to answer the question of how stress-related disorders could best be prevented as opposed to what (pharmacological) treatments could be developed to alleviate existing symptoms. Doing so, it moves away from a more traditional disease-focused approach and, instead, embraces a health-focused perspective.

To date, animal studies of resilience point to (i) the potential use of corticosterone levels as a predictor of differential susceptibility to future stress, (ii) the roles of several miRNAs in regulating vulnerability to ongoing or future stress, and (iii) the value of examining hippocampal volume and functional connectivity in a specific brainstem-limbic network as a reflection of stress vulnerability. Clinical studies highlight (i) the involvement of several genetic polymorphisms and site-specific DNA methylation patterns in susceptible phenotypes, and (ii) the use of baseline blood-based inflammatory markers as predictors of future PTSD.

The current literature on resilience points to a lack of standardization, which hampers proper scientific study of resilience. Therefore, several researchers introduced recommendations to guide future resilience studies, including the need to view resilience as a dynamic adaptation process following adversity and the need for prospective longitudinal designs in order to best capture its dynamic nature [1]. Robust resilience studies are crucial for deepening our understanding of adaptive responses to trauma, and can aid in establishing effective primary prevention protocols aimed at increasing resilience in high-risk populations, such as military members [2]. As presented in this second chapter, such interventions could include preparing at-risk individuals for potential trauma exposure through enhancing stress-management skills and applying cognitive restructuring strategies, which is much needed in at-risk populations and

currently only receives little attention within the military. Alternatively, given the critical time window between the occurrence of the traumatic event and the onset of symptoms, establishing early secondary prevention strategies are equally relevant in order to prevent the development of stress-related disorders following trauma. To date, several cognitive interventions have been tested in order to disrupt the consolidation of traumatic memories following trauma exposure, and have shown to successfully attenuate stress responses [3, 4]. Similarly, efforts aimed at promoting mindfulness or physical activities and maintaining or acquiring a strong social support network have repeatedly been shown to boost resilience and reduce PTSD-related symptoms [5].

Gaining a fundamental understanding of the biologic basis of resilience could lead to developing individualized therapies using a combination of psychotherapy and pharmacological treatments precisely aimed at targeting resilience-promoting pathways [6]. Although today, such biological underpinnings remain largely understudied and thus poorly understood, it is becoming clear that investing in prevention strategies such as the ones presented here, could increase one's psychological resilience and be beneficial for first-responders or trauma survivors in the wake of stress exposure.

Research question 2: What is known regarding the implications of microRNAs in PTSD?

MicroRNAs (miRNAs) are small non-coding RNA molecules that are mostly involved in post-transcriptional regulation of gene expression by binding to and silencing or degrading target messenger RNAs (mRNAs). Since miRNAs target mRNAs with imprecise complementarity, one miRNA can have several hundred mRNA targets and thus be involved in a large variety of biological pathways. Moreover, given their presence and stability in several biofluids, the conservation of their sequences across different species, and the relative ease with which they can be examined, miRNAs are considered to be interesting biomarker candidates [7].

As presented in **Chapter 3**, most animal studies examining miRNAs in trauma- and stress-related phenotypes, focused on (i) fear conditioning, (ii) exploring the biomarker potential of miRNAs, (iii) their implication in transgenerational stress, and (iv) their interaction with known stress-related genes such as *FKBP5*. Studies performed in humans all used military cohorts (except one), and almost all reported miRNA involvements in immune dysregulations. While most animal studies examined miRNA levels in several brain regions, all human studies focused on either whole blood or peripheral blood

mononuclear cells (PBMCs). Expression levels of three miRNAs, miR-27a-3p, miR-19b and miR-223, were found to be dysregulated in several animal models of PTSD and suffering individuals from one of the human cohorts, although not always in the same direction.

The lack of replication among studies is concerning but not entirely unexpected. Most studies used (very) small sample sizes, while the animal studies all used different techniques to induce and assess PTSD-like phenotypes. Although these studies do provide first evidence for the implication of a certain number of miRNAs in PTSD, a logical next step would be for larger studies to now build upon these reports and replicate their findings. Another critical step involves unraveling the precise functions and relevance of these circulating miRNAs since their contribution to disease is still not clear. Examining expression profiles across tissues, for example by making use of animal studies in order to examine molecular and cellular mechanisms occurring within the blood and brain, will be equally important in order to understand whether peripheral epigenetic markers reflect processes central to the etiology of PTSD. Moreover, such designs could allow for the identification of relevant and novel intervention strategies. Finally, at the time of performing the literature search, all studies used cross-sectional designs. When possible, using longitudinal designs should be encouraged in order to assess pre-existing differences in epigenetic signatures and examine dynamic fluctuations across a period of stress exposure. Using such designs will also be relevant in order to identify at-risk individuals prior to trauma exposure, i.e. individuals who are more susceptible of developing a stress-related disorder should they be exposed to a traumatic event in the future. How to best deal with this type of predictive information, is discussed further in the impact paragraph of this thesis.

Research question 3: What microRNA and DNA methylation signatures are associated with combat-related PTSD?

In **Chapter 4**, we examined circulating miRNA profiles associated with PTSD. We identified a total of 306 different miRNAs of which 22 were differentially expressed between PTSD cases and non trauma-exposed controls, and one (miR-1246) was downregulated in PTSD cases compared to resilient individuals. To assess whether clusters of miRNAs could distinguish our three groups, we used weighted gene co-expression network analysis (WGCNA). We identified three clusters of miRNAs of which one module of 79 miRNAs seemed to be both trauma- and PTSD-dependent. Its hub miRNA, miR-138-5p, was also significantly upregulated in subjects with PTSD as compared to healthy controls. This miRNA is known to be widely expressed within the

central nervous system (CNS), where it is involved in processes such as neural development, dendritic and synaptic plasticity. Expression levels of miR-138-5p have previously been found altered in a rat model of restraint stress, while hippocampal levels were associated with the formation of fear memories in mice. Evidence from human studies also points to its role as a potential regulator of memory performance.

The 79 miRNAs from the most interesting WGCNA module targeted 9270 genes. In order to perform meaningful pathway and GO enrichment analyses, this gene set was narrowed down to 146 genes, which were targeted by significantly more miRNAs than expected by chance only (using Fisher tests). This revealed involvements in several cancer- and apoptosis-related processes, of which the latter is not uncommon in PTSD and could be related to often-detected immune-related dysfunctions in PTSD. Together, the findings presented in this Chapter provide preliminary evidence for the involvement of specific miRNAs in PTSD-related phenotypes, some of which had previously been implicated in stress-related disorders. They now pave the way for future functional studies to gain insights into the precise roles of these miRNAs in stress susceptibility.

In terms of miRNAs encapsulated within exosomes, we first optimized the protocol needed to isolate neuron-derived exosomes (NDEs) from limited amounts of blood plasma (as presented in **Chapter 5**), and applied this to plasma, serum and urine samples belonging to individuals with or without PTSD in **Chapter 6**. This pilot study showed the feasibility of isolating plasma neuronal exosomes (defined as presenting the neuronal surface marker CD171), and examining encapsulated miRNA profiles. Two miRNAs, miR-542-3p and miR-581-5p, were upregulated in plasma samples from individuals with PTSD as compared to trauma-exposed matched controls. While miR-581 was previously found implicated in bipolar disorder and liver cancer, miR-542-3p was more recently found involved in major depressive disorder, Parkinson's disease and an animal model of PTSD, next to several cancer-related processes. Interestingly, pathway and gene ontology (GO) analyses using their target genes, showed enrichment in pathways and several biological processes related to hypoxia and cell damage, which provides further evidence for their potential implications in psychiatric disorders.

This first feasibility study gave room to assess whether this same protocol could also yield urine NDEs and be used on older, i.e. ~10 to 15 years old, serum samples belonging to individuals with PTSD. However, in order to assess whether such samples can be used in an efficient and informative manner, more research is needed from a technical point of view. This research should (i) assess the precise origin of CD171+ exosomes present in

urine, and (ii) explore miRNA stability in old serum samples, despite signs of sample degradation of longer RNA fragments. This will be discussed further in the sections below, along with other issues worth highlighting regarding the use of NDEs.

Chapter 7 presents one of the first longitudinal studies of DNA methylation in PTSD. We opted for a two-stage approach in which we first combined whole blood DNA methylation data from two US-based military cohorts, then sought replication in the Dutch PRISMO cohort, and finally combined all three datasets in order to increase our power to identify robust signals. When combining all three cohorts, and correcting for baseline DNA methylation levels, we identified three differentially methylated positions (DMPs) located within an intergenic site (cg05656210 located on 5q31), *MAD1L1* and *HEXDC*. We further identified 12 differentially methylated regions (DMRs), of which four were located within the immune-related human leukocyte antigen (HLA) region, and one was located within *MAD1L1*.

Since one of the cohorts had available data on single nucleotide polymorphisms (SNPs), we assessed associations between SNPs and DMPs. Interestingly, all three DMPs had significantly associated SNPs, which were located nearby and explained about 80% of the variation in methylation. Moreover, *MAD1L1* was recently identified in a PTSD genome-wide association study (GWAS) of the Million Veteran Program (MVP). The SNP that underlies the top significant probe in our findings, rs11761270, is located within the same large linkage disequilibrium block as the MVP finding. These findings point to the involvement of specific blood CpG sites in PTSD and confirm the implication of previously identified PTSD-related genes.

Together, these preliminary findings indicate that specific blood DNA methylation signatures and several miRNAs, whether or not encapsulated within exosomes, seem to be associated with PTSD. Some of these, e.g. miR-138-5p, *MAD1L1* and genes in the HLA region, along with evidence pointing to miRNA-581 and miR-542-3p involvement in hypoxia-related processes, confirm previously identified implications in trauma- or stress-related phenotypes.

Research question 4: Could the identified epigenetic signatures serve as diagnostic biomarkers of combat-related PTSD?

For a molecular signature to be a strong biomarker candidate, it needs to have high specificity (not detect many false positives) and sensitivity (detect a large number of cases

who indeed have the disorder), among other requirements [8]. Identifying a diagnostic biomarker for PTSD could have great clinical value given the high complexity of the disorder, its frequent co-morbidities, and previously mentioned stigma- and job-related issues [9]. Although extracellular miRNAs are being highly investigated for their potential to serve as biomarker candidates of disease, considerable work remains to be done. Similarly, the very first analysis of human blood neuron-derived exosomal miRNAs was done just last year, in 2019 [10]. Therefore, although this thesis presents first steps towards exploring the possibility of using epigenetic signals as markers for PTSD, this field is still very much in its infancy. While it would be too preliminary and uncalled for to talk about robust biomarkers at this stage, several aspects regarding the use of our identified epigenetic signatures as potential biomarkers are still worth pointing out.

In **Chapter 4** we explored the possibility of combining miRNAs together in order to stratify individuals based on trauma load and PTSD symptomatology. As proposed in the introduction of this thesis, combining several biological markers together, as opposed to relying on just one biological dysregulation, could be more useful when aiming to detect a robust biomarker. Applying WGCNA in this chapter represented a first step in that direction. The areas under the curves (AUCs) of the 5 miRNAs with the highest module memberships were calculated to assess their diagnostic accuracies, which were high for all miRNAs. However, these findings mostly strengthen the need of replication in larger cohorts since small sample sizes are associated with less precise Receiver Operating Characteristic (ROC) estimations [11].

In **Chapter 6**, we applied our optimized isolation and analysis protocol for NDE miRNAs, and revealed that two plasma miRNAs seem to distinguish individuals with PTSD from trauma-exposed, healthy controls in our small pilot study. Interestingly, both miRNAs were consistently detected in all PTSD samples, while being absent in all, but one, of the control samples, resulting in large AUCs. As mentioned in the previous section, further exploration is needed in order to understand whether epigenetic signatures within urine and older serum samples could also be used to distinguish PTSD individuals from controls, and larger sample sized are warranted.

Although not directly addressed in **Chapter 7**, blood-based DNA methylation signatures could also hold potential to serve as biomarkers of disease. What we know from other fields of research, e.g. cancer research, is that combining several levels of biological (including DNA methylation) dysregulations into so-called "omic"-biomarkers can be so accurate as to outperform existing clinical scores [12]. Although less explored in

psychiatry, studies on major depressive disorder (MDD) found that a set of 18 blood DNA methylation signatures associated with MDD were able to distinguish individuals with MDD from healthy controls with great sensitivity and specificity [13]. In order to verify the robustness of our detected DNA methylation signatures as biomarkers for PTSD, assessing associations with symptom presentation is not enough. Instead, the sensitivity and specificity with which they segregate individuals with PTSD from controls would need to be explored.

A final consideration regarding the use of blood biomarkers is worth mentioning. Although PTSD is mainly mediated by functional and structural aberrations within the CNS, it has also repeatedly been associated with peripheral immune and metabolic disturbances [14, 15]. Therefore, assessing (epigenetic) profiles within peripheral samples such as blood, in addition to being convenient, is also likely to mirror relevant (peripheral) disease processes, which could end up serving as easily-accessible and suitable biomarker candidates [16].

Strengths and limitations

Several strengths of the studies presented in this thesis are worth mentioning. **Chapter 5** is novel in presenting a full workflow needed to perform NDE miRNA sequencing analyses using limited amounts of biological starting material. By applying these methods, the experiments performed in **Chapters 6** are among the first to assess blood and urine NDE miRNAs, and the first to start assessing their potential relevance to PTSD. Similarly, **Chapter 7** presents the first study of its kind by incorporating baseline DNA methylation data in a combined analysis of 450k data belonging to three independent military cohorts. Another strength of these studies is their focus on combat-related PTSD specifically. It's reasonable to assume that, at least to some extent, suffering military members share similar histories of trauma, values and morals, which minimizes inter-individual variability. On the other hand, this also implies that findings from these and similar studies can perhaps not be generalized to the civilian population and likely only apply to military members who suffered during deployment.

This brings us to the main limitations of the studies presented in this thesis. The most important one is the limited sample sizes of all included studies. Two of the chapters include preliminary pilot data. While such efforts are crucial in optimizing and establishing working protocols, they limit the power of the studies to identify robust signals, and now require further validation and replication by larger independent studies.

Regarding the miRNA studies, several things need to be pointed out. First, the scientific community is still unsure about the precise function and origin of most circulating miRNAs. Although this does not necessarily mean that they would be unsuitable as biomarkers, further investigations are crucially needed. Alternatively, while examining the miRNA content of exosomes provides a valuable and interesting alternative, there currently is no standardized method to analyze exosomes. Given that some studies use ultracentrifugation, while others use a variety of commercial reagents, direct comparisons between research findings is complicated and non-replicated findings are slowly building up [17]. This is not to mention the added challenge of investigating neuron-derived exosomes. Although the approach holds great promise and is theoretically highly relevant, there currently is no consensus on how to best isolate and further characterize these vesicles [18]. This calls for protocol standardizations regarding sample preparations, study designs, analysis methods and data analysis. These steps will also make it possible to compare our generated sequencing data with what is expected from blood- or urine-derived NDEs.

A limitation of our DNA methylation study is the use of Illumina Infinium HumanMethylation450 BeadChips, which cover only a portion of all CpG sites across the genome (~ 480k) and leave methylation at non-CG sites uninvestigated. Although newer arrays such as the Illumina MethylationEPIC BeadChip are currently available, future technological developments will undoubtedly keep increasing coverage and thus allow researchers to shed light on understudied, yet relevant methylation signatures of disease. Moreover, while our sample processing included a bisulfite conversion step to identify methylation patterns, it lacks the additional oxidative step prior to the conversion, which would allow us to detect hydroxymethylation signatures [19].

When aiming to validate sequencing data, qPCRs are commonly performed. Whether or not qPCR validations are necessary given the specificity of high-throughput sequencing technologies, is up for debate, but it is worth mentioning that several qPCRs were performed in order to validate some of the miRNA findings of Chapter 4. However, our miRNAs of interest were low in expression according to the sequencing data. When performing qPCRs for these miRNAs specifically, we detected great variability between both assays (of note, this was not the case for highly expressed miRNAs). Since sample variability between assays when testing miRNAs low in abundance has been reported before [20], and given additional issues such as the restricted primer design options for miRNA qPCRs [21], and the great sensitivity of NGS technologies [22], we therefore decided to rely exclusively on our sequencing findings.

Finally, it is unlikely that just one biological signature will one day serve as a robust biomarker of a disorder as complex and multidimensional as PTSD. Instead, combining several biological levels through systems biology approaches, as previously proposed for other disorders such as major depressive disorder [23-25], and linking these with underlying (endo)phenotypes, might be more appropriate and is discussed in the next section.

Future perspectives

Several lines of research are recommended in order to move this field forward. As mentioned, the findings presented in this thesis need extensive replication and validation by independent studies using larger sample sizes. Simultaneously, systems biology approaches aimed at integrating multiple "omics" layers will be highly valuable in obtaining a more comprehensive understanding of the biological basis of PTSD. The datasets used and generated in this thesis will contribute to such efforts. Large consortia such as the Psychiatric Genomic Consortium (PGC) PTSD will further be highly useful in combining similar datasets and analyzing them jointly thanks to their standardized pipelines for quality control. Such analysis pipelines are crucial in order to deal with challenges such as population stratification (i.e. ancestry) and technical variabilities between different cohorts or sample batches.

Another important challenge is the cell heterogeneity of peripheral samples, given that epigenetic signatures are mostly cell-type specific [26]. In this thesis, and when relevant, differences in blood cell type compositions were accounted for by including estimates derived from DNA methylation data. However, emerging studies address this issue more directly by using single-cell sequencing approaches which, these coming years, will likely reveal to be better suited in detecting cell-type specific contributions to phenotypes [27].

To date, the precise functions of miRNAs are still somewhat unknown, and their interactions with mRNAs are unclear. In order to bridge this knowledge gap, both *in vitro* and *in silico* functional studies are much needed. While the former could include manipulating the expression of target miRNAs and assessing the impact on gene expression and cell morphology (among other outcomes), the latter reaches back to the previously mentioned need for multi-omics studies to investigate miRNA-mRNA interactions using available datasets.

Additionally, our understanding of the specificity of circulating miRNAs will be greatly improved once we will know where these originate from, i.e. whether they enter the circulation primarily through an active secretion process or through passive leakage due to cell injury or cell death. With cell-free DNA, determining its cell type of origin can be done by examining DNA methylation signatures specific to certain tissue types [28]. Once we know more about specific miRNA enrichments throughout different tissues in the body, the same might potentially be possible for circulating miRNAs.

Exosome research is still very much in its infancy. As we learn more about exosome isolations and analyses, valuable efforts made by the International Society for Extracellular Vesicles (ISEV), such as their publications on Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, provide general directions for exosome research and will undoubtedly be highly valuable in helping this field move forward [29]. Additionally, it will be useful to know more about the function of exosomes secreted by neurons, in order to gain a deeper understanding of their implications in the pathophysiology of brain disorders. If exosomes are indeed actively secreted by neurons as a way of intercellular communication, and if miRNAs are an integral part of this process, then understanding this signal would generate much needed knowledge regarding the (neuro)biology of PTSD, and would strengthen the use of the exosomal miRNA content as a biomarker of disease.

As mentioned before, most epigenetic studies in PTSD to date used cross-sectional designs. However, given that epigenetic mechanisms are dynamic in nature, longitudinal studies hold greater potential to unravel fluctuations in the expression of epigenetic profiles across a period of trauma exposure and/or treatment trajectory [30].

As discussed by Nievergelt et al. [31], ultimately our goal is to better understand the etiology of PTSD. Given the relative ease and relevance of examining peripheral biological fluids for biomarker discovery studies, it is valuable to keep this research line evolving. However, in order to detect biological processes centrally involved in the onset and/or progression of PTSD, future cross-tissue efforts should aim to discover whether and how blood-derived (epigenetic) markers relate to the organ of interest, in this case the brain. While assessing correlations with neuroimaging studies is one promising option [32], examining associations with molecular patterns within postmortem brain tissue might be another precious avenue worth exploring in more detail. Since postmortem tissue of individuals with PTSD and matched controls were previously scarce,

efforts such as the National PTSD Brain Bank have recently launched in the United States in order to render brain tissue available and stimulate its use in PTSD research [33].

Finally, I want to conclude with some considerations regarding the field of psychiatry in general. The current lack of clinically useable biomarkers for PTSD (or any psychiatric disorder for that matter) despite increasing research efforts, could be explained by several factors, including (i) the current classification system in psychiatry, which is primarily symptom-based and lacks validity [34], (ii) methodological limits such as the use of small sample sizes and different study assays, and (iii) the lack of usable *in vitro* models in psychiatry [35]. To tackle the first issue, the National Institute of Mental Health (NIMH) launched the Research Domain Criteria (RDoC) initiative in 2009, a research framework intended to unravel biological underpinnings of mental health and illness. The underlying motive is to re-think the current classification system in psychiatry and move away from relying on subjective symptom presentation only [36]. By mapping out (neuro)biological dimensions of behavior, health and illness, the NIMH aims to stimulate researchers to think of mental health disorders in terms of dysregulated systems as opposed to rigid symptom clusters. As this initiative will continue to grow in the coming decades, this approach will allow us to better understand the precise relationship between brain measures and behavioral outcomes in order to more accurately understand complex mental health disorders and eventually evaluate whether this could be used to establish a more appropriate diagnostic system. Additionally, given the difficulty of studying living human brains, using *in vitro* approaches to model specific aspects of psychiatric disorders using reprogrammed patient-derived cells, and thus incorporate the full genetic background of the affected individual, might be highly valuable. This approach might help us better understand some of the basic cellular and molecular mechanisms which may contribute to disease in a personalized manner [37]. Together, one could assume that such approaches driven by neuroscientific findings will eventually facilitate the identification of robust psychiatric biomarkers.

References

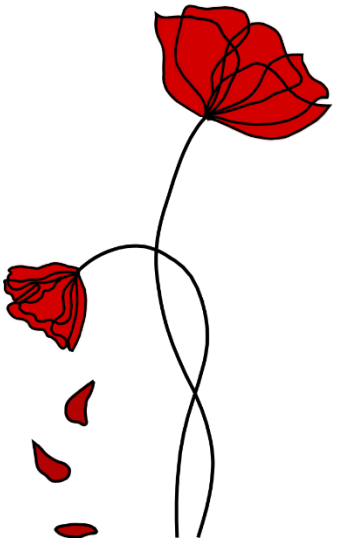
1. Kalisch, R., et al., *The resilience framework as a strategy to combat stress-related disorders.* Nat Hum Behav, 2017. **1**(11): p. 784-790.
2. Horn, S.R. and A. Feder, *Understanding Resilience and Preventing and Treating PTSD.* Harv Rev Psychiatry, 2018. **26**(3): p. 158-174.
3. Iyadurai, L., et al., *Preventing intrusive memories after trauma via a brief intervention involving Tetris computer game play in the emergency department: a proof-of-concept randomized controlled trial.* Mol Psychiatry, 2018. **23**(3): p. 674-682.
4. Holmes, E.A., C.R. Brewin, and R.G. Hennessy, *Trauma films, information processing, and intrusive memory development.* J Exp Psychol Gen, 2004. **133**(1): p. 3-22.
5. Kang, S.S., et al., *Transcendental meditation for veterans with post-traumatic stress disorder.* Psychol Trauma, 2018. **10**(6): p. 675-680.
6. Maul, S., et al., *Genetics of resilience: Implications from genome-wide association studies and candidate genes of the stress response system in posttraumatic stress disorder and depression.* Am J Med Genet B Neuropsychiatr Genet, 2020. **183**(2): p. 77-94.
7. Etheridge, A., et al., *Extracellular microRNA: a new source of biomarkers.* Mutat Res, 2011. **717**(1-2): p. 85-90.
8. Holland, R., *What makes a good biomarker?* Advances in Precision Medicine, 2016. **1**(1): p. 4-11.
9. Lehrner, A. and R. Yehuda, *Biomarkers of PTSD: military applications and considerations.* Eur J Psychotraumatol, 2014. **5**.
10. Cha, D.J., et al., *miR-212 and miR-132 Are Downregulated in Neurally Derived Plasma Exosomes of Alzheimer's Patients.* Front Neurosci, 2019. **13**: p. 1208.
11. Min, L., et al., *Evaluation of circulating small extracellular vesicles derived miRNAs as biomarkers of early colon cancer: a comparison with plasma total miRNAs.* J Extracell Vesicles, 2019. **8**(1): p. 1643670.
12. Zhu, B., et al., *Integrating Clinical and Multiple Omics Data for Prognostic Assessment across Human Cancers.* Sci Rep, 2017. **7**(1): p. 16954.
13. Numata, S., et al., *Blood diagnostic biomarkers for major depressive disorder using multiplex DNA methylation profiles: discovery and validation.* Epigenetics, 2015. **10**(2): p. 135-41.
14. Wang, Z., B. Caughron, and M.R.I. Young, *Posttraumatic Stress Disorder: An Immunological Disorder?* Front Psychiatry, 2017. **8**: p. 222.
15. Michopoulos, V., A. Vester, and G. Neigh, *Posttraumatic stress disorder: A metabolic disorder in disguise?* Exp Neurol, 2016. **284**(Pt B): p. 220-229.
16. Nievergelt, C.M., et al., *Genomic Approaches to Posttraumatic Stress Disorder: The Psychiatric Genomic Consortium Initiative.* Biol Psychiatry, 2018. **83**(10): p. 831-839.
17. Yang, D., et al., *Progress, opportunity, and perspective on exosome isolation - efforts for efficient exosome-based theranostics.* Theranostics, 2020. **10**(8): p. 3684-3707.
18. Hornung, S., S. Dutta, and G. Bitan, *CNS-Derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges.* Front Mol Neurosci, 2020. **13**: p. 38.
19. Stewart, S.K., et al., *oxBS-450K: a method for analysing hydroxymethylation using 450K*

BeadChips. Methods, 2015. **72**: p. 9-15.

20. Chen, Y., et al., *Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis*. BMC Genomics, 2009. **10**: p. 407.
21. Ouyang, T., et al., *MicroRNA Detection Specificity: Recent Advances and Future Perspective*. Anal Chem, 2019. **91**(5): p. 3179-3186.
22. Coenen-Stass, A.M.L., et al., *Evaluation of methodologies for microRNA biomarker detection by next generation sequencing*. RNA Biology, 2018. **15**(8): p. 1133-1145.
23. Papakostas, G.I., et al., *Assessment of a multi-assay, serum-based biological diagnostic test for major depressive disorder: a Pilot and Replication Study*. Molecular Psychiatry, 2013. **18**(3): p. 332-339.
24. Pajer, K., et al., *Discovery of blood transcriptomic markers for depression in animal models and pilot validation in subjects with early-onset major depression*. Transl Psychiatry, 2012. **2**: p. e101.
25. Redei, E.E., et al., *Blood transcriptomic biomarkers in adult primary care patients with major depressive disorder undergoing cognitive behavioral therapy*. Transl Psychiatry, 2014. **4**: p. e442.
26. Bauer, M., *Cell-type-specific disturbance of DNA methylation pattern: a chance to get more benefit from and to minimize cohorts for epigenome-wide association studies*. Int J Epidemiol, 2018. **47**(3): p. 917-927.
27. Tang, X., et al., *The single-cell sequencing: new developments and medical applications*. Cell Biosci, 2019. **9**: p. 53.
28. Wan, J.C.M., et al., *Liquid biopsies come of age: towards implementation of circulating tumour DNA*. Nature Reviews Cancer, 2017. **17**(4): p. 223-238.
29. Thery, C., et al., *Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines*. J Extracell Vesicles, 2018. **7**(1): p. 1535750.
30. Ryan, J., et al., *Biological underpinnings of trauma and post-traumatic stress disorder: focusing on genetics and epigenetics*. Epigenomics, 2016. **8**(11): p. 1553-1569.
31. Ratanatharathorn, A., et al., *Epigenome-wide association of PTSD from heterogeneous cohorts with a common multi-site analysis pipeline*. Am J Med Genet B Neuropsychiatr Genet, 2017. **174**(6): p. 619-630.
32. Di Battista, A.P., et al., *Blood biomarkers are associated with brain function and blood flow following sport concussion*. J Neuroimmunol, 2018. **319**: p. 1-8.
33. Friedman, M.J., et al., *VA's National PTSD Brain Bank: a National Resource for Research*. Curr Psychiatry Rep, 2017. **19**(10): p. 73.
34. Brunoni, A.R., *Beyond the DSM: trends in psychiatry diagnoses*. Archives of Clinical Psychiatry (São Paulo), 2017. **44**: p. 154-158.
35. Venkatasubramanian, G. and M.S. Keshavan, *Biomarkers in Psychiatry - A Critique*. Ann Neurosci, 2016. **23**(1): p. 3-5.
36. Sanislow, C.A., et al., *Advancing Translational Research Using NIMH Research Domain Criteria and Computational Methods*. Neuron, 2019. **101**(5): p. 779-782.
37. Maze, I., et al., *Analytical tools and current challenges in the modern era of neuroepigenomics*. Nat Neurosci, 2014. **17**(11): p. 1476-90.

CHAPTER 9

Summary



The aims of this thesis were to (i) examine how studying resilience factors could be beneficial for deepening our understanding of trauma susceptibility, and (ii) gain insights into epigenetic underpinnings of post-traumatic stress disorder (PTSD) and start exploring whether some of these could potentially serve as diagnostic biomarkers for PTSD. In order to answer these questions, this thesis presented two literature reviews, three experimental studies and one methodological chapter.

First, the available literature on resilience studies was summarized in a review presented in **Chapter 2**. This overview highlights the need to standardize resilience studies, starting with the way resilience is currently being defined. Studying factors that could promote resilience, either through cognitive strategies or pharmacologically by targeting biological pathways of resilience, if and when identified once study designs are optimal, will be beneficial for at-risk individuals. This is further emphasized by the fact that addressing potential mental health issues is currently not always receiving sufficient attention within the military.

In order to get a first understanding of what is known regarding the implications of microRNAs (miRNAs) in PTSD, a second review was presented in **Chapter 3**. By being easily accessible and relatively stable in biofluids and across species, miRNAs hold the exciting potential to serve as biomarkers of disease. In the context of PTSD, most of the work has been done in animals by modeling traumatic stress and studying associated phenotypes, while most human research examined trauma-related miRNAs in military populations. These studies are mostly characterized by small sample sizes and a great variety in the way miRNAs are being analyzed. Although informative as a first exploration of miRNA dysregulations in PTSD, we suggest that future studies use longitudinal designs by incorporating time points prior to and following stress exposure in order to gain insights into the role of circulating miRNAs in the onset and course of PTSD. When possible, combining such studies with animal studies would be of added value in order to track corresponding molecular mechanisms occurring within the brain which could potentially yield novel avenues for treatment strategies.

Next, several experimental studies were performed using blood samples from deployed military members. In **Chapter 4** we performed a first pilot study in order to examine circulating miRNA profiles of deployed military members with or without PTSD. Expression profiles of several miRNAs were found to be dysregulated between individuals with PTSD, trauma-exposed controls and healthy controls, some of which had previously been associated with PTSD. Although preliminary, this study highlights the feasibility and

potential usefulness of clustering miRNAs based on their expression profiles and use this information to distinguish individuals with PTSD from (trauma-exposed) healthy controls.

This study gave rise to several questions, one of which included whether exosomal miRNAs would be better suited for sampling and analysis given the higher stability of encapsulated miRNAs and the potential that these vesicles offer to track their tissue of origin. The protocol needed to isolate and analyze exosomal miRNAs within plasma neuron-derived exosomes (NDEs), i.e. exosomes released by neurons which end up in the blood circulation, was presented in **Chapter 5**. This methodological chapter provides insights into the workflow needed to capture these vesicles and sequence their miRNA content.

In **Chapter 6**, we then applied this knowledge to plasma samples belonging to individuals with PTSD, along with urine samples from the same individuals, and serum samples from an independent cohort of PTSD subjects. This study is the first to assess the miRNA content of NDEs present in limited amounts of several human biofluids using high-throughput sequencing. The findings generated by this pilot study indicate several aspects. First, it is feasible to sequence the miRNA content of NDEs using small amounts of human plasma. Next, two specific miRNAs could potentially, if replicated in larger cohorts, serve as markers for PTSD, especially given the interesting fact that both were expressed in all samples of PTSD subjects, while being absent in all (but one) of the control samples. More research is needed in order to assess whether urine can be used for these analyses, for example by assessing the precise origin of L1CAM+ exosomes present in urine. Finally, our findings suggest that older serum samples might still be useful for NDE miRNA analyses, although the stability of these miRNAs should be assessed further given the potentially considerable degradation of larger RNA fragments.

Finally, in **Chapter 7** we took a different approach of looking at epigenetic mechanisms in PTSD and focused on DNA methylation by performing one of the first studies that used longitudinal measures of DNA methylation. We combined DNA methylation data from three military cohorts from which blood methylation and phenotypic information was collected prior to and following combat exposure. Our findings highlight several differentially methylated positions and regions, some of which had previously been associated with PTSD, thereby enhancing their potential implication in the disorder.

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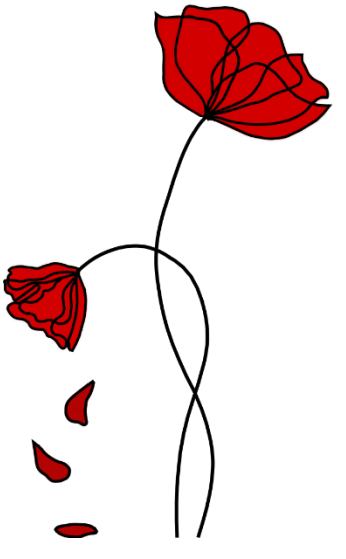

In this section, the scientific and societal impact of my research will be clarified.

Scientific impact

Although post-traumatic stress disorder (PTSD) is a highly debilitating psychiatric disorder, no medical tools are currently available to prevent or minimize the impact of traumatic stress on mental health. Moreover, PTSD remains difficult to treat, with the only currently FDA-approved pharmacological treatment options being two antidepressants [1]. There is thus a pressing need to identify precise (neuro)biological mechanisms mediating risk and resilience to the effects of traumatic stress in order to better understand its biological basis, which in turn could lead to more optimal treatment strategies.

The studies presented in this thesis aim to unravel biological underpinnings of PTSD, some using novel study designs. For example, sequencing the microRNA (miRNA) content of human blood and urine neuron-derived exosomes has not been done yet so far. Chapters 6 therefore presents some of the first studies assessing such miRNA profiles using limited amounts of starting material. Studies like these, along with the optimized protocol needed to analyze these miRNAs, which is presented in Chapter 5, contribute to establishing the scientific foundation this field needs by striving for more standardization. Next, using longitudinal DNA methylation data as opposed to cross-sectional data only is another relatively unexplored and novel avenue representing an interesting advancement in the field by yielding potentially more specific methylation patterns due to correcting for pre-existing differences. The study presented in Chapter 7 is among the first to perform these type of analyses in relation to PTSD.

The generated data can now be embedded within larger systems biology efforts aiming to combine several biological layers in order to deepen our understanding of PTSD. This highlights the scientific relevance of the present studies, even though the findings should be regarded as preliminary and are in need of future replication and validation using larger study cohorts. The acquired knowledge could further give rise to a range of follow-up studies, including functional analyses of the identified miRNAs, *in silico* studies to predict miRNA-messenger RNA (mRNA) interactions, *in vitro* studies using patient-derived cells in order to observe dynamic cellular behavior upon administration of stress-related (exosomal) miRNAs, and extrapolation of the findings to other cohorts in order to verify overlap with stress-related disorders other than PTSD. Moreover, as epigenetic modifications are in principle reversible, robust alterations could prove to be interesting targets for therapeutic interventions in the future.

Anticipated societal impact

The economic burden of PTSD is substantial [2]. Since trauma exposure is close to inevitable in certain populations such as deployed military members, the prevalence of PTSD among war veterans is far greater than in the general population [3]. The symptoms associated with PTSD prevent suffering individuals from leading a healthy lifestyle and are debilitating on a personal, societal as well as a professional level [4]. Since establishing a PTSD diagnosis is mostly based on self-reported symptoms, this thesis encourages the development of a biological test in order to obtain a more precise and accurate reflection of PTSD status. As mentioned before, while returning military members are at high risk of developing PTSD, the current stigma associated with mental health disorders could prevent some of them from seeking appropriate healthcare [5]. Moreover, with PTSD being classified as a "mental" health disorder, they may not be willing to fully disclose their symptoms out of concerns for other people's opinions or job-related consequences. Therefore, and given the added difficulty of diagnosing PTSD given its complexity and high rates of co-morbidities, using objective biomarkers would be highly beneficial as an addition to post-deployment clinical assessments. Identifying such a marker could then encourage suffering individuals to seek treatment faster, and optimize their chances of returning to a "normal" lifestyle as soon as possible, thereby going back to contributing to society. Importantly, although not addressed directly here, the search for such a biomarker should go alongside efforts aiming to destigmatize disorders such as PTSD and change the narrative surrounding mental health disorders in general, which is especially crucial in populations such as the veteran population.

At this stage, the findings presented in this thesis are still too preliminary to result in any concrete, finished or clinically usable product. However, once validated, replicated, and perhaps embedded within a broader network of biological dysregulations associated with PTSD, the identified epigenetic markers could hold potential to serve as diagnostic biomarkers. Their presence in blood further makes them ideal biomarker candidates given the relative ease with which blood can be drawn. Additionally, examining the utility of using such markers as predictors of treatment response on an individual level could be interesting in yielding recommendations for personalized treatment strategies. However, this has to be viewed in light of the current limitations of identifying psychiatric biomarkers, as detailed in Chapter 8.

The populations that would benefit from the research presented in this thesis, are military members returning from combat, or individuals with similar high-risk professions.

Inversely, for military members who are about to be sent out, resilience studies presented in this thesis show that several preventive (cognitive) interventions could be implemented during pre-deployment preparation periods in order to increase one's psychological resilience. Gaining deeper insights into the biologic basis of resilience and resilience-promoting approaches such as mindfulness, could lead us to develop personalized preparation steps and/or treatment plans, potentially by combining psychotherapeutic strategies and pharmacological treatments precisely aimed at targeting resilience-promoting pathways [6].

Although the search for psychiatric biomarkers is steadily increasing, practical, societal or even legal implications of using and interpreting such biomarkers, have been given little attention. As discussed by Lehmer and Yehuda (2014) [5], the search for a PTSD biomarker should be accompanied by interdisciplinary discussions in order to understand how to best incorporate potential biomarkers within clinical settings. Undoubtedly, bioethicists should join the conversation in order to weigh in on the ethical implications of using such markers. Importantly, the complexity of PTSD should under no circumstances be reduced to the mere presence or absence of certain markers. How to best understand and use biomarkers to benefit suffering individuals, and how this information should be translated to society, needs critical attention. Similarly, the extent to which biomarkers developed for combat-related PTSD in the military will be applicable to civilian populations, will also need further examination [7].

References

1. Alexander, W., *Pharmacotherapy for Post-traumatic Stress Disorder In Combat Veterans: Focus on Antidepressants and Atypical Antipsychotic Agents.* P T, 2012. **37**(1): p. 32-8.
2. McGowan, l., *The Economic Burden of PTSD. A brief review of salient literature.* International Journal of Psychiatry and Mental Health, 2019. **1**(1): p. 20-26.
3. Lehavot, K., et al., *Post-traumatic Stress Disorder by Gender and Veteran Status.* Am J Prev Med, 2018. **54**(1): p. e1-e9.
4. Kessler, R.C., *Posttraumatic stress disorder: the burden to the individual and to society.* J Clin Psychiatry, 2000. **61 Suppl 5**: p. 4-12; discussion 13-4.
5. Lehrner, A. and R. Yehuda, *Biomarkers of PTSD: military applications and considerations.* Eur J Psychotraumatol, 2014. **5**.
6. Maul, S., et al., *Genetics of resilience: Implications from genome-wide association studies and candidate genes of the stress response system in posttraumatic stress disorder and depression.* Am J Med Genet B Neuropsychiatr Genet, 2020. **183**(2): p. 77-94.
7. Yehuda, R., et al., *The use of biomarkers in the military: from theory to practice.* Psychoneuroendocrinology, 2013. **38**(9): p. 1912-22.

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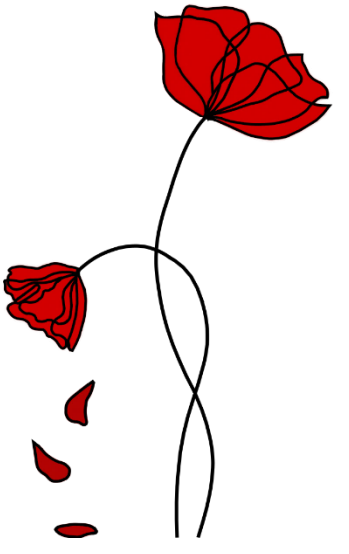

Clara Snijders was born on November 18th 1992, in Brussels, Belgium. Initially intrigued by the intricate functioning of the mind, she started her Bachelor studies in Psychology at the Vrije Universiteit Brussel in 2010. Three years later, she decided to obtain a Master degree in Clinical Psychology with a specialization in Biological Psychology at the same university. During her Master internship, she worked with traumatized children and adolescents who were in need of a neuropsychological assessment and rehabilitation. What fascinated her most was the incredible plastic nature of the developing brain, which fueled her motivation to study this organ on a molecular level. She obtained her Master degree *magna cum laude*, and was accepted to the Fundamental Neuroscience track of the Research Master Cognitive and Clinical Neuroscience at Maastricht University in The Netherlands. For her internship, Clara joined the group of Prof. dr. Bart Rutten at the same university, and was introduced to the world of epigenetics and post-traumatic stress disorder (PTSD) under supervision of Dr. Laurence de Nijs. Ongoing collaborations with the University of California in San Diego (UCSD), led her to complete part of her internship in the lab of Dr. Caroline Nievergelt. She graduated *cum laude* in 2017, and continued this line of research as a PhD student under supervision of Prof. dr. Bart Rutten, Dr. Laurence de Nijs and Dr. Gunter Kenis. During her PhD, Clara collaborated with UCSD and the Veterans Affairs hospital of San Diego, where she spent 8 months performing exosome and microRNA research under primary supervision of Dr. Nievergelt. Her work was presented at various conferences, including the Society of Biological Psychiatry for which she obtained the 2017 Top Poster award for Basic Research. Upon completion of her PhD, Clara will continue her scientific career as a postdoctoral researcher at the McLean Hospital of the Harvard Medical School, in collaboration with Maastricht University.

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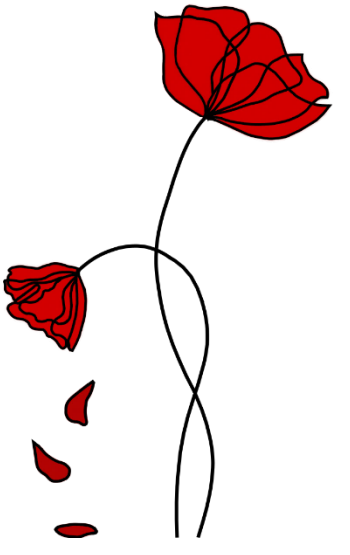

Published

Research articles

- **Snijders, C.**, Maihofer, A.X., Ratanatharathorn, A., Baker, D.G., Boks, M.P., Geuze, E., Jain, S., Kessler, R.C., Pishva, E., Risbrough, V.B., Stain, M.B., Ursano, R.J., Vermetten, E., Vinkers, C.H., PGC PTSD EWAS Consortium, Smith, A.K., Uddin, M., Rutten, B.P.F., Nievergelt, C.M. Longitudinal epigenome-wide association studies of three male military cohorts reveal multiple CpG sites associated with post-traumatic stress disorder. *Clinical Epigenetics,* 12(11). (2020)

- Sillivan, S.E., Jamieson, S., de Nijs, L., Jones, M., **Snijders, C**., Klengel, T., Joseph, N.F., Krauskopf, J., Kleinjans, J., Vinkers, C.H., Boks, M.P.M., Geuze, E., Vermetten, E., Berretta, S., Ressler, K.J., Rutten, B.P.F., Miller, C.A. MicroRNA regulation of persistent stress-enhanced memory. *Molecular Psychiatry*, 25: 965-976. (2020)

- **Snijders, C.**, Krauskopf, J., Pishva, E., Eijssen, L., Machiels, B., Kleinjans, J., Kenis, G., van den Hove, D., Myeong Ok, K., Boks, M.P.M., Vinkers, C.H., Vermetten, E., Geuze, E., Rutten, B.P.F., de Nijs, L. Circulating serum microRNAs as potential diagnostic biomarkers of post-traumatic stress disorder: a pilot study. *Frontiers in Genetics,* 10(1042). (2019)

- Agorastos, A., Hauger, R.L., Barkauskas, D.A., Lerman, I.R., Moeller-Bertram, T., **Snijders, C.**, Haji, U., Patel, P.M., Geracioti, T.D., Chrousos, G.P., Baker, D.G. Relations of combat stress and posttraumatic stress disorder to 24-hour plasma and cerebrospinal fluid interleukin-6 levels and circadian rhythmicity. *Psychoneuroendocrinology,* 100: 237-245. (2019)

Review articles and book chapters

- Pries, L-K, **Snijders, C.,** Menne-Lothmann, C., Decoster J., van Winkel, R., Collip, D., Delespaul, P., De Hert, M., Derom, C., Thiery, E., Jacobs, N., Wichers, M., Guloksuz, S., van Os, J., Rutten, B.P.F. TwinssCan - gene-environment interaction in psychotic and depressive intermediate phenotypes: risk and protective factors in a general population twin sample. *Twin Research and Human Genetics,* 22(6): 460-466. (2019)

- **Snijders, C.**, Pries, L-K., Sgammeglia, N., Al Jowf, G., Youssef, N.A., de Nijs, L., Guloksuz, S., Rutten, B.P.F. Resilience against traumatic stress: current developments and future directions. *Frontiers in Psychiatry,* 9(676). (2018)

- **Snijders, C.**, Bassil, K., de Nijs, L. Methodologies of neuroepigenetic research: background, challenges and future perspectives. *Progress in Molecular Biology and Translational Science,* 158: 15-27. (2018)

- **Snijders, C.**, de Nijs, L., Baker, D.G., Hauger, R.L., van den Hove, D., Kenis, G., Nievergelt, C.M., Boks, M.P., Vermetten, E., Gage, F.H., Rutten, B.P.F. MicroRNAs in post-traumatic stress disorder. *Current Topics in Behavioral Neurosciences,* 38: 23-46. (2018)

- Zwamborn, R.A.J., **Snijders, C.**, Ning. A., Thomson, A., Rutten, B.P.F., de Nijs, L. Wnt signaling in the hippocampus in relation to neurogenesis, neuroplasticity, stress and epigenetics. *Progress in Molecular Biology and Translational Science*, 158: 129-157. (2018)

Abstract publications

- De Nijs, L., Krauskopf, J., **Snijders, C**., Kleinjans, J., Machiels, B., Smeets, B., Kenis, G., Van den Hove, D., Vinkers, C., Geuze, E., Boks, M., Vermetten, E., Rutten, B.P.F. Circulating microRNAs as potential biomarkers of differential susceptibility to traumatic stress. *European College of Neuropsychopharmacology*. (2017)

- Logue, M., **Snijders, C.**, Maihofer, A., Rutten, B.P.F., Miller, M. The Traumatic Stress Brain Study Group, Uddin, M., Nievergelt, C., Smith, A. GWAS-implicated genes inform cross-tissue studies of DNA methylation. *Biological Psychiatry,* 87(9), S53. (2020)

In preparation

- **Snijders, C.**, de Nijs, L., Baker, D.G., Hauger, R.L., van den Hove, D., Kenis, G., Nievergelt, C.M., Boks, M.P., Vermetten, E., Gage, F.H., Youssef, N.A., Rutten, B.P.F. MicroRNAs in stress-related disorders. *Invitation to contribute a chapter to Epigenetics of Stress and Stress Disorders. In preparation.*

- Schowe, A., **Snijders, C.** *et al.* Biomarkers in stress-related disorders: potential, challenges and future directions of blood-based diagnostics. *In preparation.*

- Al Jowf, G., **Snijders, C.**, Eijssen, L.M.T, de Nijs, L., Rutten, B.P.F. The molecular biology of susceptibility to PTSD: highlights of epigenetics and epigenomics. *In preparation.*

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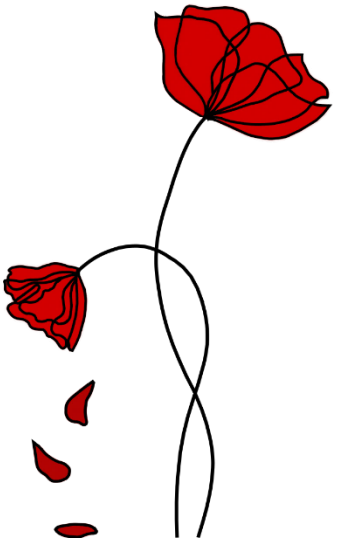

I want to start this section off by thanking my supervisory team. Dear Bart, a couple years ago I was lucky enough to start my Master's internship under your supervision. During one of our first meetings, you casually mentioned there might be a possibility for me to go to San Diego for a few months. Although that day was probably not memorable to you, it was to me. And sure enough, a few months later I was tanning on a beach in Calif-euh, working hard in the lab! Thank you for always thinking big, for seeing possibilities and opportunities instead of set-backs and obstacles. Thank you for encouraging me to discover what I want to achieve in these next chapters of my life, all the while being readily available to provide tons of valuable advice and never-ending support.

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Caroline, while I thank Bart for sending me away in 2017, I thank you for welcoming me into your lab! A couple years have passed since, but I still want to thank you for always being willing to troubleshoot on the many lab failures (oh boy, the flashbacks), for repeatedly managing to break down my messy thoughts into clear goals and action points, and for being easily approachable to discuss projects and future career steps. Whether for personal of professional reasons, I knew/know your door was/is always open and I very much appreciate that.

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Having arrived at the end of my PhD, I want to conclude with some final thoughts regarding these last few years. During my studies, I never thought I would one day embark upon, let alone finish, a PhD project. Although I certainly was interested in research, I did not think I had what it takes. Working through those thoughts now brings me here, to this very last section of my thesis, in which I want to thank myself (yes, Snoop Dogg inspired me to write this). I want to thank myself for not giving up, even after months of challenging and failing experiments. For persevering, despite every so often wanting to drop everything. For recognizing my fears and facing them anyways. For challenging myself and for increasingly believing in myself.

To any fellow PhD student relating to this; make sure you give yourself enough credit for occasionally wondering why you are putting up with those failing experiments and messy datasets, but persisting anyways. As the American psychologist B.F. Skinner put it;

A failure is not always a mistake. It may simply be the best one can do under the circumstances. The real mistake is to stop trying.